white paper

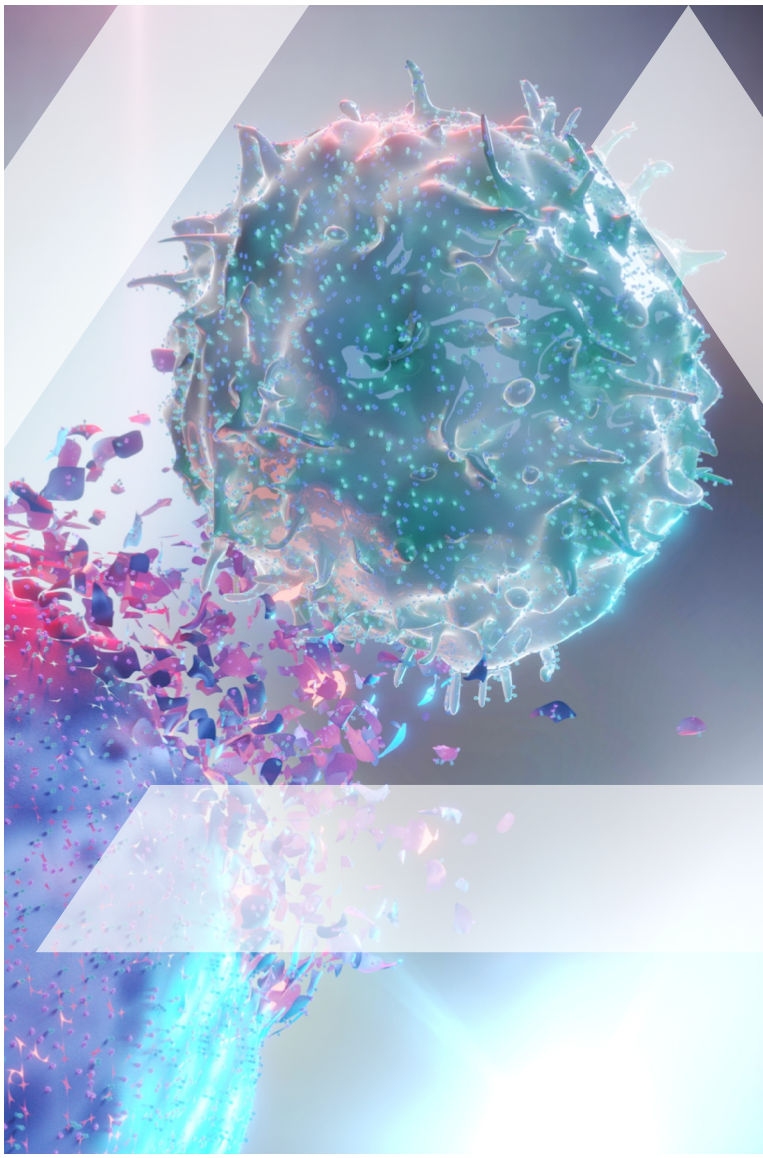
Rapid and Efficient Therapeutic Antibody Discovery Using AvantGen's Germliner™ Library Collection

Key Takeaways:

- Therapeutic antibodies specific to CD16a, a natural killer cell marker targeted in bispecific NK cell engager therapy, are particularly difficult to develop due to high sequence similarities with CD16b on other immune cells.

- By employing AvantGen's large yeast display library of over 100 billion clones, rationally designed to reflect the natural diversity of human antibodies, highly specific CD16a antibody clones were discovered within four months.

- Robustly functional anti-CD16a antibodies with excellent engager potency were identified and shown to possess highly developable characteristics: high affinity, high specificity, high stability, and low immunogenicity.



Bispecific natural killer cell engager (NKCE) therapy represents a promising approach in cancer immunotherapy. Using sequences from two fragment antigen-binding (Fab) or single-

chain variable (scFv) antibodies, a recombinant bi-specific antibody can be constructed with affinity for both an NK cell target and a cancer cell marker.[1,2] The result is an NK cell tethered to a cancer cell, resulting in activation of the NK cell and killing of the cancer cell by NK cell-mediated antibody dependent cellular cytotoxicity (ADCC) and other mechanisms of action.[3]

One of the most attractive NK cell targets for NK cell engager development is CD16a, because it mediates both ADCC and cytokine production, promoting recruitment of other immune cells. But there's a catch: a surface marker with 98.5% sequence identity, CD16b, is found on neutrophils. Because neutrophils are abundant and CD16b has been observed to negatively regulate ADCC, an antibody targeting CD16a for NKCE therapy must have no affinity for its nearly identical cousin.[4] Conventional methods for generating monoclonal antibodies (mAbs) with this level of specificity and functionality are time consuming, labor intensive, and nearly impossible.

Yet, a panel of mAbs was generated in only 4 months, with high affinity to CD16a, ultra-high specificity, excellent stability, and high potency under physiological conditions.[5] The rapid and efficient discovery of these candidates was made possible using a unique yeast display platform featuring a fully human, rationally designed Fab fragment library of over 100 billion clones.

Immunization and phage display antibody screening strategies fall short for difficult antigen targets

Standard strategies for monoclonal antibody discovery rely on immunization or phage display-based screening, but each method brings limitations that can result in sub-optimal antibody candidates that are found unsuitable for their application during development.[6] When dealing with challenging targets, such as CD16a, this not only extends the development timeline, but risks program termination.

Immunization-based monoclonal discovery is time consuming and labor-intensive, so limited antibody pools are used for screening and may not include candidates with the highest possible affinity and specificity. Candidates must be further refined and optimized to meet development criteria.

Phage display strategies improve efficiency by screening orders of magnitude more antibodies, but the bacteria used to generate these libraries do not fold or modify antibodies with the same mechanisms as mammalian cells. A common result is antibody candidates that initially show binding in the screening process, but function poorly when expressed in production cell lines, such as CHO cells, which can lead to significantly higher development costs. By the same token, an antibody that might have performed well when expressed in mammalian cells can be missed entirely when screened by phage display.

These limitations are particularly concerning when developing an anti-CD16a antibody for use in NKCE therapy. In addition to issues with cross-reactivity against CD16b, which may be present as one or both of two alleles, NA1 and NA2, monoclonals targeting CD16a face additional hurdles. CD16a also has two common alleles, 176V and 176F, and therapeutic antibodies must have affinity for both. Plus, it is desirable for the antibody to have affinity for cynomolgus monkey CD16a (cyno CD16a) to facilitate preclinical, nonhuman primate testing.

So, how do you generate an antibody with high affinity to three closely related surface markers, but no affinity to a fourth and fifth, without spending years in discovery? Utilizing a more rapid, powerful, and optimized platform for antibody discovery is the answer.

How Germliner™ selects antibodies with highly developable properties

The AvantSabre platform, part of the Germliner™ Library Collection developed by AvantGen, features a rationally designed and fully synthetic library with over 100 billion fully human Fab fragment clones.

Most antibody libraries that are synthetically modified to increase diversity contain a high number of clones with problematic properties or reduced functionality, due to deleterious substitutions not present in the general population. However, Germliner libraries were designed using a proprietary human antibody database that amassed >500 individuals' antibody repertoires, and are weighted toward rearrangements at heavy and light chain sites with high natural diversity and variation from germline sequence in the CDRs **(Figure 1)**, while keeping the framework regions 100% germline. The result is a library that reflects the structures and functionalities of human antibodies, enabling discovery of clones

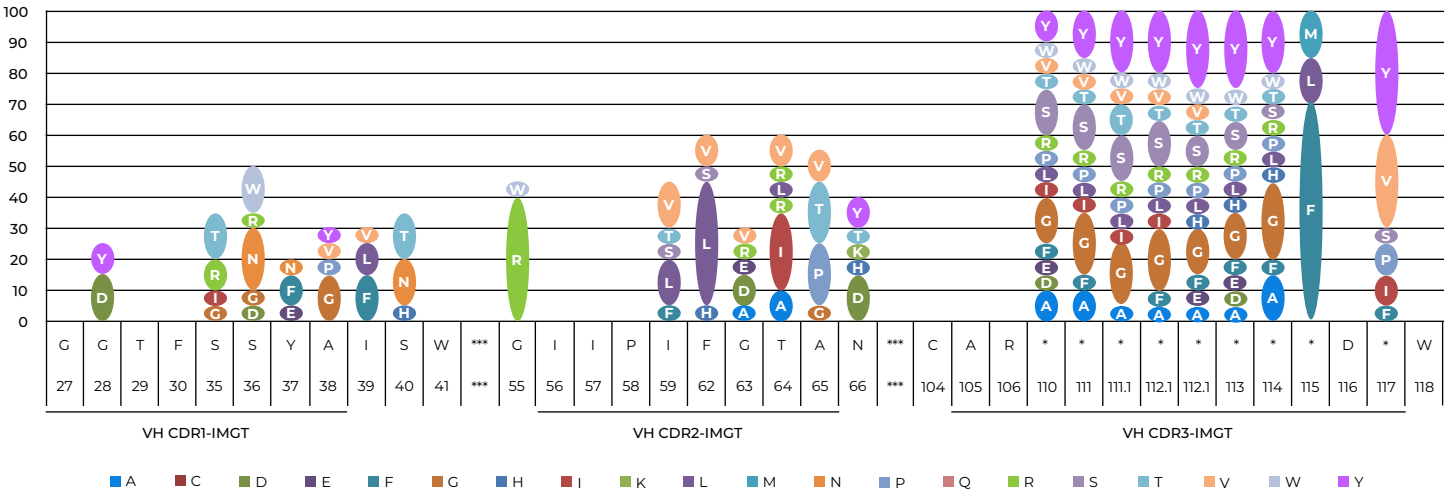


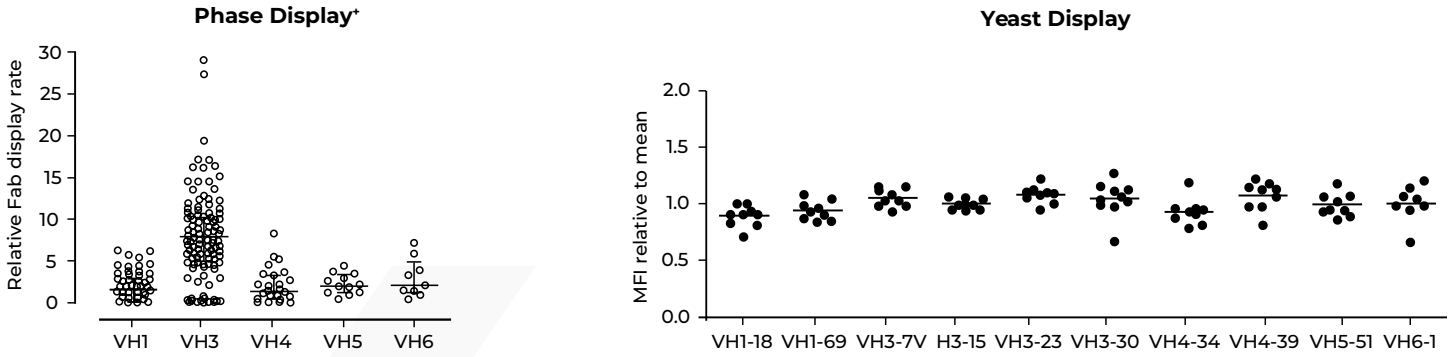
Fig 1. Distribution of heavy chain CDRs for a representative variable domain family, with germline amino acids and common variants shown at each position on the x-axis, and variation frequency on the y-axis. Informed by deep sequencing of over 500 individuals, rearrangements in Germliner™ libraries are weighted towards positions and amino acids that mimic natural sequence variation in human antibodies, thus reducing immunogenicity and increasing developability.

with easily developable characteristics: high affinity, high specificity, high stability, and low immunogenicity.[7]

Additionally, antibodies are produced in and displayed on yeast. Yeast has similar protein folding and quality control mechanisms as mammalian cells, so antibodies discovered with a yeast display platform are less likely to suffer a drop in expression or affinity when produced in mammalian cell lines, or be missed during the initial screen due to non-biased display **(Figure 2)**.[7]

Display System	Antibody Format	Diversified Library Display	Biological Relevance	Developability
Phage	Limited	Limited/Biased	Low	Low
Yeast	Extensive	Excellent	High	High

Diversified Library Display in Phage vs. Yeast Systems



⁺Adapted from Morphosys; mAbs 5:3, 445-70; 2013

Fig 2. Comparison of *in vitro* antibody discovery platforms. Due to display efficiency and eukaryotic expression, folding, and post-translational mechanisms, yeast display produces antibody candidates in extensive formats, with equal display across all major variable region families, resulting in optimal screening and higher biological relevance and developability when expressed in mammalian cells.

How the screening and selection process works

Anti-CD16a antibody discovery with AvantGen's AvantSabre platform began with biotinylated CD16a-176V and CD16a-176F antigens introduced to yeast cells displaying the Fab library. Yeast cells displaying antibodies with affinity for these markers were enriched using magnetic-activated cell sorting (MACS) with avidin-coated magnetic beads. This reduced the candidate pool from 100 billion to 100 million clones after two sequential rounds.

Additional screening was performed using fluorescent-activated cell sorting (FACS). Selecting for the best CD16a-176V and CD16a-176F binders brought the pool down to 10,000. Selecting against CD16b's NA1 and NA2 alleles and a polyspecificity reagent further reduced the candidate pool to around 100. This portion of the screening process required less than 2 months **(Figure 3)**.

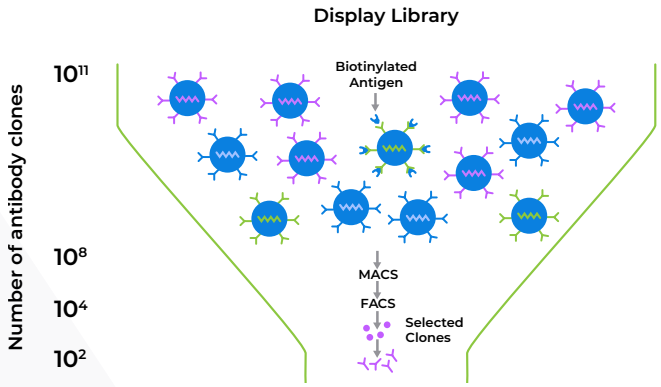


Fig 3. The ease and power of MACS sorting combined with quantitative FACS sorting reduced the antibody pool to a manageable number of strong candidates in less than 2 months. Enriched candidates were screened by ELISA and flow cytometry.

AvantGen's novel display system independent of Aga1-Aga2 utilizes an optimized anchoring and secretion peptide switch dependent on different media conditions. Final screening was streamlined by inducing secretion of antibody candidates directly from yeast clones isolated in the previous steps. Antibodies were then assessed by ELISA and flow cytometry analysis of CD16a- and CD16b-expressing 293F cells stained with candidate antibodies. This step was completed in under 2 weeks. Top candidates were then recombinantly produced and further characterized. As a result of this workflow, 5 candidates with excellent characteristics as therapeutic monoclonal antibodies were identified.

Kinetic analysis showed that all 5 clones had outstanding affinity to desired targets CD16a-176V, CD16a-176F, and Cyno-CD16; and high specificity, as demonstrated by low to no binding to CD16b NA1 and NA2 **(Figure 4)**. These clones were also tested by a competition assay, and shown to share an epitope with an anti-CD16a IgG1 reference antibody currently in clinical trial (Reference), but not to a pan-CD16a/b control antibody.[5]

Neutrophil binding was tested in a subset of these candidates, using cultured primary neutrophils from donors with both the NA1 and NA2 alleles of CD16b. No binding was detected in clone A2-3.[5] Epitope analysis by site-directed mutagenesis of CD16a showed no binding to a Y158H CD16a variant, demonstrating that the single tyrosine (Y) at position 158 is solely responsible for their high specificity.[5]

Clone A2-3 was further tested by flow cytometry for binding to primary NK cells, and found to bind similarly to Reference. It also exhibited better thermostability and its binding was less attenuated under physiological concentrations of IgG compared to Reference.[5]

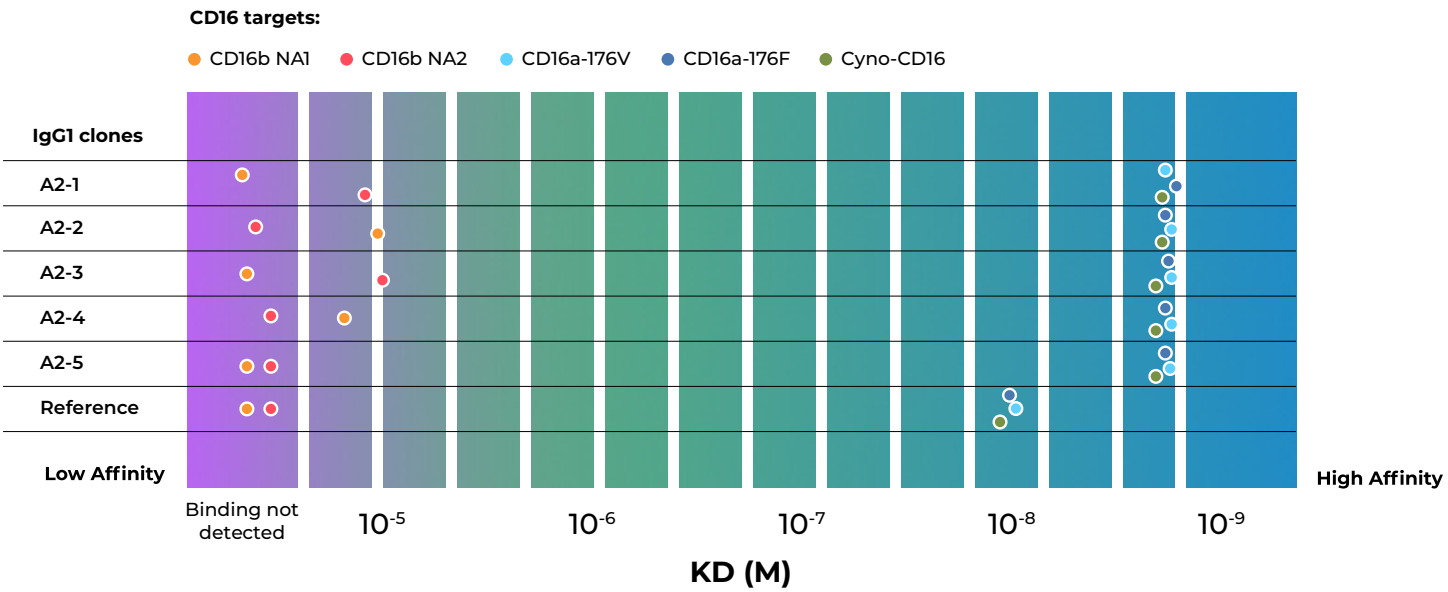


Fig 4. Equilibrium dissociation constant (KD) of top monoclonal antibody candidates to CD16 targets. All candidates display high affinity and specificity. Reference is an IgG1 CD16a-specific antibody currently in clinical trial.

Bispecific engager activity of antibody panel

Sequences for this panel of 5 clones were used for construction of bispecific antibodies, with anti-CD16a coupled to an anti-CD19 antibody in both tandem scFv and bivalent tandem scFv-IgG4 formats **(Figure 5a)**. These were expressed in Expi293F™ cells.

In the bivalent tandem format, these antibodies were found to be potent in activating Jurkat cells expressing CD16a-176V, in the presence of lymphoblast-like, CD19-expressing Raji cells.

Clones A2-1 and A2-3 were more potent, in fact, than the same anti-CD19 as a monoclonal antibody with an ADCC-enhanced engineered Fc (Fc enhanced) that is currently used in clinical trials. Furthermore, A2-1 and A2-3 potency is retained even in the presence of physiological simulating conditions of 10mg/ml IgG, while both Fc enhanced and Reference coupled with anti-CD19 (Bispecific reference) lose almost all of their activity **(Figure 5b)**. When no Raji cells are present, no activation is detected, demonstrating that both targets must be bound for activation to occur, and thus that Jurkat cells were activated by bispecific engager activity.

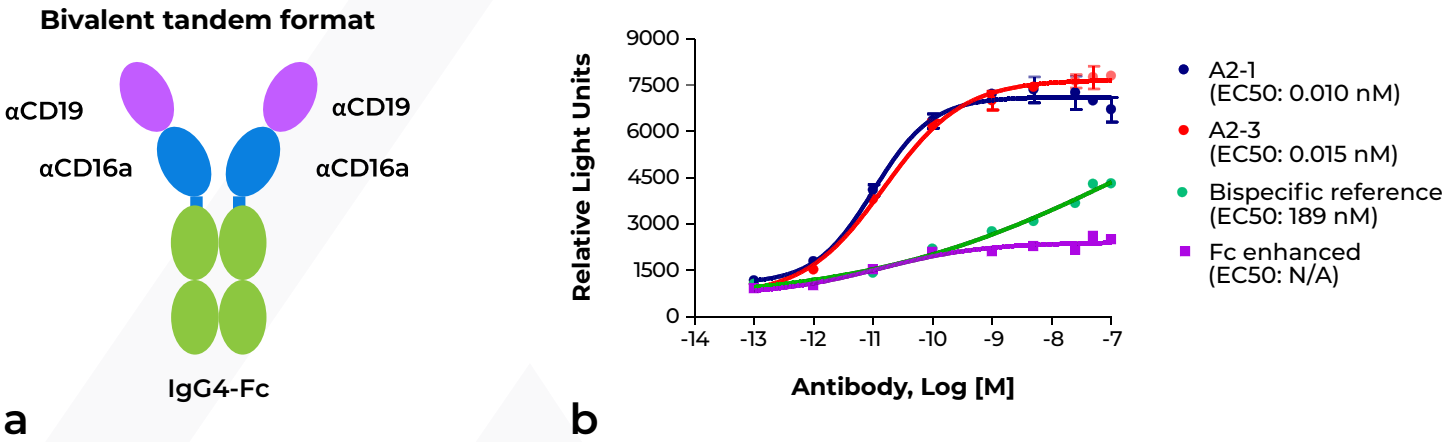


Fig 5. (a) Bivalent tandem scFv-IgG4 format (CD19-CD16-IgG4-Fc). (b) In a reporter assay, A2-1 and A2-3 bispecific engagers activate an engineered CD16a-expressing Jurkat cell line in the presence of CD19-expressing Raji cancer cells, in physiologically relevant conditions of 10mg/ml IgG. Their potency is significantly more than ADCC Fc-enhanced and Bispecific reference controls.

A primary NK cell cytotoxicity assay showed that clone A2-3 efficiently promotes killing of Raji cells at an EC50 value of 100pM or lower, in the presence of 10mg/ml IgG **(Figure 6)**. This represents superior performance compared to Bispecific reference. Furthermore, in the tandem scFv format, clones A2-1 and A2-3 showed cytotoxic activity with EC50 values of around 5pM, which was one order of magnitude more potent than Bispecific reference.[5]

In short, after only months of screening and development, a panel of antibodies identified with the AvantSabre platform matched or exceeded the performance of a leading clinical-stage reference antibody to CD16a, across multiple biophysical properties and a range of formats and assays.

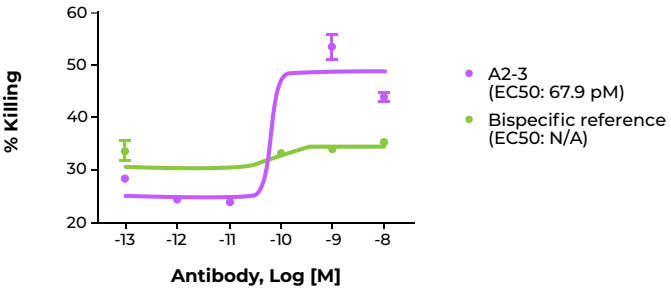


Fig 6. Primary NK cell cytotoxicity assay shows efficient killing of Raji cells by bispecific A2-3 in physiologically relevant conditions of 10mg/ml IgG. In contrast, Bispecific reference loses potency in the presence of 10mg/ml IgG.

Fast development of therapeutic antibody lead candidates

Conventional therapeutic antibody discovery against challenging targets produces candidates which are, at best, suitable only after a time-consuming cycle of optimization for affinity, specificity, stability, and robust functionality under physiological conditions.[6] At worst, poor developability characteristics in antibody candidates emerging from discovery can result in termination of the project.

However, highly specific antibody-based therapeutics with a shorter development cycle are possible with Germliner and AvantSabre. By utilizing an extensive rationally designed, one-of-a-kind fully human antibody library built along lines of natural diversity, and a novel and robust yeast display system, antibody candidates are pre-optimized for development into therapeutic drugs.

By harnessing the power of rational design and yeast display, Germliner represents a new era in antibody discovery—one defined by speed, specificity, and revolutionary therapeutic potential.

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