

***In vitro* maturation of affinity of CD antibodies by CHO cell display**

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Abstract

Cluster of Differentiation (CD) markers serve as critical tools for immune cell classification in flow cytometry. However, domestic diagnostic antibody manufacturers in China have long been constrained by reagent quality limitations, which hinder their competitiveness against well-established international suppliers. To tackle this bottleneck, we employed a Chinese hamster ovary (CHO) cell display-based *in vitro* evolution system to optimize the enhanced cellular detection and stability of antibodies targeting CD33 and CD56. Compared with the parental antibody clones, the resultant mutant clones exhibited significantly enhanced sensitivity in detecting peripheral blood leukocytes, alongside improved stability. This study provides a feasible strategy for elevating the quality of CD-specific antibodies, with the potential to bolster high-quality domestic antibody production and advance diagnostic performance across a wide spectrum of CD markers.

Keywords

Cluster of Differentiation (CD), antibody, CHO cells, *in vitro* evolution, CD33, CD56, affinity, stability

1. Introduction

Cluster of Differentiation (CD) numbers are assigned to panels of monoclonal antibodies (mAbs) that recognize specific molecules expressed on the surface of human leukocytes and the other types of cells. The CD nomenclature system has been universally adopted by the International Human Leucocyte Differentiation Antigen (HLDA) Workshop, which has designated a total of 408 CD molecules to date (Engel *et al.* 2015). A specific cell type displays a unique cluster of multiple cell-surface molecules that reflects its differentiation stage, and such cells can be precisely identified via multi-color flow cytometry in combination with diverse fluorescence-conjugated antibodies. CD marker proteins typically function as receptors or ligands, and a number of CD proteins are involved in critical cellular processes including cell signaling, cell adhesion, cell inhibition, and cell activation (Arora and Tomar 2024). CD molecules are widely utilized as cell surface markers, enabling the accurate identification and isolation of distinct leukocyte populations, subpopulations, and cells at different differentiation stages. The identification and enumeration of immune cells using antibodies targeting CD molecules represent indispensable tools in clinical diagnosis and biomedical research (Clark *et al.* 2016). Additionally, several CD molecules serve as therapeutic targets for various diseases, and antibodies directed against these targets have been approved as clinical therapeutic agents (Shouse 2025; Yasui *et al.* 2025).

Antibody affinity can be enhanced via rational design, CDR-targeted mutagenesis, and display-based screening platforms. Multiple display technologies, including phage, ribosome RNA, bacterial, yeast, and mammalian cell display, have been widely applied to antibody discovery and affinity maturation workflows. Phage and yeast display systems remain the most prevalent choices, owing to the relative simplicity of antibody library construction and the superior robustness of their screening and selection pipelines. By contrast, mammalian cell display recapitulates native eukaryotic expression machinery, which more accurately recapitulates authentic antibody folding, secretion, post-translational modifications, and expression-associated developability profiles (Slavny *et al.* 2024).

Antibodies specific to CD molecules play a pivotal role in clinical diagnosis, disease prognosis assessment, and biomedical research (Gorczyca 2022; Shapiro 2003), driving the rapid growth of the global market for these antibodies. Although the market size of CD

diagnostic antibodies in China reached 3.5 billion RMB in 2024 (Grand View Research 2023, 2024), domestic manufacturers have failed to secure a substantial market share, primarily due to quality constraints of locally produced antibody reagents. In this study, we report the successful enhancement of cellular detection and stability of antibody for two clinically significant CD targets, CD33 and CD56, using a CHO cell display-based *in vitro* evolution system initially established in Hang's Lab (Chen *et al.* 2016; Luo *et al.* 2022; Luo *et al.* 2020). Compared with the parental antibody clones, the engineered antibody variants exhibited significantly improved sensitivity in detecting peripheral blood leukocytes. These findings demonstrate a feasible and effective strategy for optimizing the performance of CD-specific diagnostic antibodies, laying a solid foundation for upgrading the quality of domestic CD antibody reagents and boosting the competitiveness of domestic diagnostic antibody manufacturers in the global market.

2. Materials and methods

2.1 Plasmid construction

Two substitution plasmids encoding human CD33 single-chain variable fragment (hCD33 scFv) and human CD56 single-chain variable fragment (hCD56 scFv) antibodies were designated as pFRT-*hCD33* scFv-loxP (abbreviated as pFCD33L) and pFRT-*hCD56* scFv-loxP (abbreviated as pFCD56L), respectively. The anti-hCD33 and anti-hCD56 single-chain antibodies were derived from clone My9-6 and huN901, respectively (Hoffe *et al.* 2008; Wang *et al.* 2005). These plasmids were utilized to insert the *hCD33* scFv and *hCD56* scFv genes into a specific chromosomal locus in heat-resistant CHO cells for subsequent *in vitro* antibody evolution. FRT and loxP represent two recombinase recognition sites, which are employed to exchange the *hCD33* scFv and *hCD56* scFv genes with the puromycin (Puro) resistance gene in CHO cells (Chen *et al.* 2016). Both of the single-chain antibodies are high-expression mutants generated following sequence optimization by GenScript Biotech Corporation (Nanjing, China). Notably, this optimization process did not alter the encoded amino acid sequences, but solely modified the DNA sequences to boost protein expression levels, thereby improving the mutation efficiency of hCD33 scFv and hCD56 scFv during *in vitro* evolution. The two plasmids pFCD33L and pFCD56L were constructed by replacing the α TNF α gene in the pFRT- α TNF α -

HA-loxP plasmid between the *Bam*HI and *Bgl*II restriction endonuclease sites. HA refers to the hemagglutinin tag, which was fused to the N-termini of hCD33 scFv and hCD56 scFv to facilitate the monitoring of their expression levels on the surface of CHO cells. The parental pFRT- α TNF α -HA-loxP plasmid was from Hang's laboratory (Chen *et al.* 2016).

The plasmid pCDNA3.1-*hCD33* scFv-HA-TM was constructed by inserting the hCD33 scFv-HA fragment into the pCDNA3.1-TM vector between the *Nhe*I and *Xho*I restriction endonuclease sites. TM denotes the transmembrane domain, which serves to anchor the hCD33 scFv-HA fusion protein onto the cell surface. Analogous plasmids for the transient expression of mutant hCD33 scFvs, wild-type hCD56 scFv, or mutant hCD56 scFvs were constructed using the identical molecular cloning strategy.

The hCD33-scFv-Fc2a fusion antibody was applied for the detection of human peripheral leukocytes and stability assessment in this study. The recombinant antibody plasmid pCDNA3.1-*hCD33*-scFv-Fc2a was generated by inserting the *hCD33-scFv* gene into the pCDNA3.1-Fc2a vector (obtained from Hang's lab, where Fc2a represents the fragment crystallizable region of mouse IgG2a) at the *Hind*III and *Kpn*I restriction sites. This plasmid drives the expression of a single-chain antibody fused with the mouse IgG2a Fc region. Likewise, the pCDNA3.1-*hCD56*-scFv-Fc2a plasmid, which expresses the hCD56-scFv-Fc2a fusion antibody, was constructed via the same cloning procedure.

2.2 Cell culture

CHO/dhFr⁻ (dihydrofolate reductase-deficient Chinese hamster ovary cells) and a series of cell lines established on the basis of CHO/dhFr⁻ cells were cultured in IMDM medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The complete culture medium was formulated by supplementing the basal medium with 10% fetal bovine serum (FBS, Hyclone; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 $\times$ HT supplement, and 4% penicillin-streptomycin double antibiotics at a final concentration of 100 U/mL. All cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO₂. For *in vitro* antibody evolution, heat-resistant CHO cells (Luo *et al.* 2022) displaying the mutant anti-CD33 or anti-CD56 scFv antibody library were incubated at 41 °C for 24 hours prior to flow cytometric sorting, aiming to enrich high-quality mutant antibody variants.

293F cells were cultured in FreeStyle™ 293 Expression Medium (Sino Biological Inc., Beijing, China) and maintained in shake flasks at 37 °C with 5% CO₂ and orbital shaking at 125 rpm.

2.3 Cell Transfection

2.3.1 Transfection of CHO Cells

- (1) One day prior to transfection, seed cells into six-well plates at a density of 2.5×10^5 cells per well using routine IMDM medium devoid of double antibiotics, to ensure the cells reach 70% confluency at the time of transfection.
- (2) Preparation of transfection complexes: Label two Eppendorf (EP) tubes as Tube A and Tube B. Slowly add 5 µL of Lipofectamine™ 2000 (Invitrogen Corporation, Carlsbad, CA, USA) to 150 µL of Opti-MEM serum-free medium in Tube A, and incubate the mixture at room temperature for 5 minutes. Dilute 2 µg of the target plasmid in 150 µL of Opti-MEM serum-free medium in Tube B and mix gently. Subsequently, slowly add the diluted plasmid mixture from Tube B to the diluted Lipofectamine™ 2000 solution in Tube A, mix thoroughly by gentle pipetting, and incubate the combined mixture at room temperature for 25 minutes to form transfection complexes.
- (3) Aspirate the original cell culture medium, wash the cells three times with 800–1,000 µL of Opti-MEM medium per wash, then evenly add the freshly prepared transfection complexes to each well of the cell culture plate. Supplement each well with an additional 500 µL of Opti-MEM medium for subsequent culture.
- (4) After 5.5 to 6 hours of incubation, replace the culture medium with routine antibiotic-free medium and continue culturing the cells.

2.3.2 Transfection of 293F Cells

- (1) One day before transfection, collect a small aliquot of the cell suspension for cell counting. Dilute the cell suspension with fresh culture medium to a density of 0.8×10^6 cells/mL. Secure the cap of the culture flask tightly and incubate it on an orbital shaker for overnight culture. Transfection was performed 12 hours later.

- (2) Dilute the target plasmid with OptiPRO™ SFM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). For every 100 mL of cell culture medium, dilute 150 µg of plasmid DNA with 6 mL of OptiPRO™ SFM. Mix the solution gently and incubate at room temperature for 5 minutes.
- (3) Add 200 µL of Sinofection transfection reagent (Sino Biological Inc., Beijing, China) to the diluted DNA solution, mix thoroughly by gentle inversion or pipetting, and incubate the mixture at room temperature for 15 minutes to allow transfection complex formation.
- (4) Add the transfection complex mixture dropwise to the cell culture suspension. Swirl the culture flask gently to ensure uniform mixing, tighten the flask cap, and place the flask back on the orbital shaker to continue cell culture.

2.4 Protein Expression and Purification

2.4.1 His-Tagged Protein Purification

Plasmids harboring the *CD33-His* or *CD56-His* fusion gene were transfected into 293F cells utilizing FreeStyle™ MAX Transfection Reagent. Following transfection, the cells were cultured on an orbital shaker for 6 consecutive days to facilitate recombinant protein expression. The cell culture was first centrifuged at $350 \times g$ for 3 minutes to discard the cell pellet, and the protein-containing culture supernatant was collected. The collected supernatant was further subjected to high-speed centrifugation at $10,000 \times g$ for 30 minutes to remove residual cellular debris and medium impurities.

Subsequently, the clarified supernatant was concentrated 20-fold via centrifugation at 3,800 rpm and 4 °C using a 10 kDa molecular weight cutoff ultrafiltration concentrator. The concentrated protein solution was loaded onto a Sepharose High Performance chromatography column (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) pre-charged with nickel sulfate (NiSO_4) for affinity purification. Bound proteins were eluted sequentially with phosphate buffers supplemented with gradient concentrations of imidazole (30, 60, 90, 120, and 250 mM, respectively). The eluted protein fractions were collected, further concentrated using an ultrafiltration tube, and the purification efficiency and protein integrity were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4.2 Antibody Affinity Purification

Plasmids encoding the target antibody gene were transfected into 293F cells with FreeStyle™ MAX Transfection Reagent. Post-transfection, the transfected cells were cultured on an orbital shaker for 6 days to allow sufficient antibody expression. The cell culture was centrifuged at $350 \times g$ for 3 minutes to remove the cell pellet, and the supernatant containing the recombinant antibody was collected for subsequent processing. The collected supernatant was further centrifuged at $10,000 \times g$ for 30 minutes to eliminate residual cellular debris and medium impurities, followed by filtration through a $0.45 \mu m$ filter membrane to obtain a clarified sample. The filtered supernatant was then concentrated to a suitable volume using a 30 kDa membrane cassette, and the concentrated sample was subjected to affinity purification with a protein A column. Prior to sample loading, the protein A column was equilibrated with Binding buffer (0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2). The concentrated sample was loaded onto the equilibrated column three times to maximize antibody binding. Unbound impurities were thoroughly washed away with Binding buffer, and the bound target antibody was eluted with Elution buffer (0.1 M citric acid, pH 2.7). The collected eluate was immediately neutralized to pH 7.0 with Neutralizing buffer, then further concentrated using a 30 kDa ultrafiltration tube. Finally, the purification efficiency and integrity of the purified antibody were verified by protein gel electrophoresis.

2.5 Flow Cytometry Detection and Sorting

Flow cytometry detection and cell sorting were conducted using a FACS AriaIII flow cytometer (BD, Franklin Lakes, NJ, USA). Fluorescent dyes FITC and APC were excited by 488 nm and 630 nm lasers, respectively. CHO cells displaying wild-type or mutant anti-CD33 scFv were labeled with His-CD33 protein (Sino Biological Inc., Beijing, China) followed by anti-His-FITC antibody (Abcam plc, Cambridge, UK). For CHO cells expressing anti-CD56 scFv, labeling was performed using His-CD56 protein (Sino Biological Inc., Beijing, China) in combination with anti-His-FITC antibody.

For flow cytometry sorting, cells were harvested via trypsinization, washed once with PBS, and resuspended in Opti-MEM medium at a density of approximately 2×10^7 cells/mL. His-tagged CD antigens at varying concentrations (adjusted per experimental requirements) were

added, and the cell suspension was incubated at 42 °C for 30 minutes for antigen binding. After centrifugation to remove the unbound antigens, cells were stained with anti-HA-APC antibody (BD, Franklin Lakes, NJ, USA) at a 1:200 dilution and anti-His-FITC antibody at a 1:300 dilution, followed by one wash with Opti-MEM. The stained cells were resuspended for subsequent sorting on the FACS AriaIII. For flow cytometry detection, the same protocol was applied, except that the cell labeling step was performed at 37 °C for 30 minutes instead of 42 °C.

For flow cytometric analysis of antibody detection sensitivities, human peripheral blood samples were prepared following the procedure below:

- (1) Collect 100 μ L of human peripheral blood.
- (2) Add fluorescence-labeled antibodies, mix thoroughly, and incubate the mixture at room temperature for 15 minutes in the dark.
- (3) Add 2 mL of 1 $\times$ FACS lysing solution, vortex gently for a short time, and incubate at room temperature for 10 minutes in the dark. Centrifuge the sample at 300 $\times$ g for 5 minutes, then discard the supernatant.
- (4) Wash the cell pellet once with 1 mL PBS, centrifuge at 300 $\times$ g for 5 minutes, and remove the supernatant.
- (5) Resuspend the cells in 200 μ L PBS for flow cytometric analysis.

APC-conjugated anti-CD33 and anti-CD56 antibodies were purchased from Beijing Quantobio and BioLegend, respectively. The in-house anti-CD33 and anti-CD56 antibodies were labeled with APC dye (Wuhan Liangdong Technology Co., Ltd., Wuhan, Hubei, China) following the manufacturer's standard labeling protocol.

2.6 Antibody Structural Modeling

No publicly resolved crystal or NMR structures are available for the two parental antibodies or their cognate antibody–antigen complexes. This impedes mechanistic dissection of pivotal structural alterations induced by amino acid mutations that govern enhanced antigen binding affinity and thermal stability. Conventional mainstream antibody structure prediction tools, including IgFold (Ruffolo *et al.* 2023), DeepAb (Ruffolo *et al.* 2022) and ImmuneBuilder (Abanades *et al.* 2023), largely remain focused on static snapshots. Although algorithms

dedicated to conformational landscape analysis have been reported (Wayment-Steele *et al.* 2024; Xiang *et al.* 2026), they are predominantly tailored for monomeric single-chain proteins and fail to accurately characterize intricate interchain interactions and synergistic conformational coordination between antibody heavy and light chains. Accordingly, we employed our self-developed antibody-specific deep-learning platform capable of constructing physiologically realistic conformational ensembles (Zong *et al.* 2026). Structural modeling of wild-type and mutant anti-CD33 and anti-CD56 antibodies was performed to elucidate conserved conformational variations underlying improved antigen-binding capacity and structural stability.

3. Results

3.1 *In vitro* evolution for improvement of cellular detection and stability of antibody

Although numerous published studies, including our own work, demonstrate that display-based affinity maturation effectively enhances antibody affinity, the present study aims only to establish a straightforward, cost-effective workflow for improving antibody cellular detection performance and stability. We do not seek to quantify the extent to which gains in cellular detection stem from enhanced antibody affinity. Accordingly, cellular detection, rather than affinity, will serve as the readout where appropriate.

In this work, anti-CD33 (My9-6) and anti-CD56 (huN901) antibodies were utilized as model templates for *in vitro* affinity maturation. Our mammalian cell-based *in vitro* evolution platform, reported previously (Luo *et al.* 2022), comprises three sequential experimental stages described below.

First, single-chain variable fragment (scFv) coding sequences were integrated into an expression cassette positioned at a fixed chromosomal locus within a thermotolerant CHO cell line (**Figure 1**). This heat-resistant CHO host strain, specialized for antibody evolution to simultaneously improve antibody cellular detection and stability, was constructed in our earlier work (Luo *et al.* 2022). To generate the S0 cell population, CHO cells were transfected with plasmids encoding CD33 or CD56 scFv and cultured for expansion. Given heterogeneous surface antibody display across transfected cells, fluorescence-activated cell sorting (FACS) was performed to enrich clones with high scFv surface expression. The sorted cell fraction was further amplified and named the S0 population.

Second, engineered activation-induced cytidine deaminase (AID) was expressed in CHO cells to trigger robust mutagenesis of antibody coding sequences. Cells were incubated at 41 °C and expanded to a total population exceeding 1×10^8 , generating a diverse mutant antibody library through the accumulation of sequence variants during cell division. Flow cytometry demonstrated that a subpopulation within the anti-CD33 mutant library displayed markedly elevated binding to fluorophore-conjugated CD33 antigen compared to cells expressing wild-type My9-6 scFv (**Figure 2A**), confirming the emergence of high-cellular detection variants relative to the parental antibody. Comparable enhancement of antigen-binding capacity was detected in a subpopulation of the anti-CD56 mutant library (**Figure 2B**). Roughly 8×10^7 library cells were stained and processed via FACS; approximately 6,000 cells with the top combined scFv expression and antigen-binding signals were isolated and propagated at 41 °C (**Figure 3**). This sorted expanded cell pool was designated as S1 population.

Third, a batch of approximately 8×10^7 S1 mutant library cells was stained and sorted by flow cytometry, completing a second full round of *in vitro* evolution consisting of AID plasmid transfection, 41 °C proliferative culture (**Figure 3**), and FACS enrichment of clones with maximal surface antibody display and strongest binding to fluorophore-labeled CD33 or CD56. The resulting sorted cells were defined as the S2 population. Mutated antibody genes were amplified and cloned from S2 cells, and 50 independent clones for each antibody target were subjected to Sanger sequencing to identify recurrent enriched mutations (**Table 1**). Sequencing results showed that four unique amino acid substitutions were enriched across both heavy and light chains in 76% of anti-CD33 clones, whereas 24% of sequenced clones retained the wild-type sequence. For anti-CD56, only a single variant harboring two amino acid mutations was recovered, with wild-type sequences accounting for 48% of all sequenced clones. Further detailed evolutionary metrics associated with our selection strategy can be found in our previous publication (Luo *et al.* 2020) and Luo's doctoral dissertation (Luo, 2019).

3.2 Examination of the antigen-binding activity and stability of the mutated antibody gene clones

All mutant antibody genes (**Table 1**) and their parental wild-type genes were transiently expressed in CHO cells. Subsequently, the cells were labeled to assess antibody display and

antigen-binding activity (**Figure 4A and 4B**). The geometric means (GMean) of antibody display levels and antigen-binding activities were quantified and presented in **Table 2, Figure 4C and 4D**, respectively. All mutant anti-CD33 antibodies exhibited superior binding to CD33 protein compared to the wild-type antibody, indicating that the two rounds of *in vitro* evolution successfully enhanced cellular detection (**Figure 4C**). Additionally, all mutant anti-CD33 antibodies except the S75N clone demonstrated higher display levels than their corresponding wild-type counterpart. Generally, a higher antibody display level is positively correlated with enhanced antibody stability.

The *in vitro* evolution strategy was designed to enhance both the cellular detection and stability of the antibody. The enrichment of the S75N mutation may likely be attributed to its ability to improve cellular detection, albeit at the expense of stability. In clones harboring the S75N mutation in combination with other point mutation(s), S75N contributed to the improvement in antigen-binding activity, while its undesirable impact on antibody display was compensated by the other mutation(s). This resulted in mutant clones with a net gain in both cellular detection and stability. In contrast to the multiple type mutant clones, only one type mutation clone was generated via the *in vitro* evolution process (**Figure 4D**).

Antibody stability is typically positively correlated with its display level on the cell surface; this correlation, however, requires further validation via measurement of the protein's melting temperature. Here, we utilized the UNcle Protein Stability Analysis Platform to compare wild-type and mutant clones of both anti-CD33 and anti-CD56 antibodies. Specifically, the mutant clones with the highest antigen-binding activities—anti-CD33 (S75N+L185V+S206N) and anti-CD56 (A104G+S189T)—were selected for comparison (**Figure 5**). Protein size analysis was conducted via protein gel electrophoresis (**Figure 5A**). Notably, in contrast to the wild-type antibody, both mutants exhibited minimal background signals adjacent to their main protein bands, indicating improved stability of mutant proteins during storage and experimental handling. Furthermore, the main band intensity of the mutant anti-CD33 protein was significantly higher than that of its wild-type counterpart, suggesting enhanced resistance to degradation and aggregation. Interestingly, the mechanisms underlying stability improvement differed between the two mutants (**Figure 5B and 5C**). For anti-CD33, the stabilization effect spanned a broad range from 25 °C to above 60 °C, implying that the wild-type was vulnerable

not only to high temperatures but also to mild thermal challenges. In contrast, for anti-CD56, the mutations (A104G+S189T) primarily enhanced stability within the range of 43 °C to 67 °C, albeit with minor improvements below 43 °C. The melting temperatures (T_m) of wild-type and mutant anti-CD33 antibodies were determined to be 56.2 °C and 60.6 °C, respectively, while those of wild-type and mutant anti-CD56 antibodies were 57.9 °C and 63.2 °C (**Figure 5D**).

3.3 Structural modeling to reveal molecular mechanisms underlying enhanced performance of mutant antibodies

Publicly available structural data for neither the two parental antibodies nor their corresponding antibody–antigen complexes have been documented. Accordingly, we constructed structural models of both wild-type and mutant antibodies using our recently established deep-learning modeling approach (Zong *et al.* 2026), and visualized diverse conformational ensembles for each antibody variant (**Figure 6**).

All three mutations identified in anti-CD33 were located at framework regions flanking CDRH2–CDRH3 (Ser→Asn), CDRL1–CDRL2 (Leu→Val), and CDRL2–CDRL3 (Ser→Asn). These substitutions markedly narrowed the conformational diversity of antibody variable regions, particularly within CDRH1, CDRL1, CDRH3 and CDRL3. This indicates that unfavorable flexible conformations were eliminated, while dominant conformations favorable for antigen binding were preserved and stabilized. Restricted conformational heterogeneity further demonstrates improved structural rigidity and thermodynamic stability of the mutant anti-CD33 antibody.

Distinct from framework mutations in anti-CD33, the two mutations in anti-CD56 resided directly within complementarity-determining regions, namely Ala→Gly in CDRH3 and Ser→Thr in CDRL2 (**Figure 6C and D**). Mutations predominantly altered the spatial conformation and outward orientation of CDR loops. Conformational states of CDRH3 were reduced, whereas CDRH2 exhibited elevated conformational diversity alongside an evident shift in spatial projection. Distinct structural alteration patterns between anti-CD33 and anti-CD56 revealed that two independent molecular mechanisms could concurrently drive enhanced antigen binding and thermal stability.

3.4 Comparison of Antibody Sensitivity in Detecting Human Peripheral Blood Cells

We aimed to generate highly sensitive antibodies for the detection of human peripheral leukocytes with flow cytometry. To this end, mutant antibodies (scFv-Fc) were conjugated with Allophycocyanin (APC) and evaluated alongside commercially available counterparts.

For anti-CD33, all mutant anti-CD33 antibodies exhibited superior leukocyte-binding performance compared to GMP-AB-00135, a product from Beijing QuantoBio Biotech—one of China’s leading flow cytometry reagent manufacturers (**Figure 7**). Flow cytometry analysis revealed that the cell populations labeled with mutant antibodies formed tighter, more cohesive clusters with markedly stronger signal intensities compared to those labeled with the commercial antibody (**Figure 7A**). This enhanced signal strength was further quantified and confirmed by their higher geometric mean (GMean) values of antigen-binding activity (**Figure 7B**).

For anti-CD56, a direct comparison was performed between our mutant anti-CD56 antibody and the commercial antibody from BioLegend (Catalog #318332). As illustrated in **Figure 8A**, the wild-type antibody generated significantly weaker signals, with its signal profile overlapping closely with the background noise, in contrast to the clear separation achieved by the BioLegend antibody. Notably, the mutant antibody outperformed both the wild-type and the BioLegend antibody, yielding stronger signal intensities. This superior performance of the mutant antibody was substantiated by the higher GMean values observed in the quantitative analysis (**Figure 8B**).

4. Discussion

Here, we report a significant enhancement in both detection sensitivity and stability for two antibodies, anti-CD33 and anti-CD56, achieved through a robust CHO cell-based *in vitro* evolution platform (**Figures 5, 7B, 8B**) (Luo *et al.* 2022). The lead mutants derived from this directed evolution process exhibit superior sensitivity in detecting human peripheral leukocytes compared to their commercially available counterparts. This work establishes a pivotal technological pathway for elevating the quality of domestically developed anti-CD antibodies and enables Chinese biotech firms to challenge the market share currently dominated by a small number of international reagent giants within the Chinese market.

Our findings demonstrate a positive correlation between the spatial separation of target cells (CD33⁺ or CD56⁺) and unrelated non-target cells (CD33⁻ or CD56⁻) and the resultant detection sensitivity. Specifically, all optimized anti-CD33 mutants display a broader window of target-to-non-target cell separation than the commercial antibodies (**Figure 7A**). Furthermore, quantitative analysis based on the geometric mean of CD56-binding activities reveals that the mutant anti-CD56 antibody offers a wider separation interval between CD56⁺ and CD56⁻ cells than the commercial antibody from BioLegend, Inc. Notably, this represents a significant improvement over the original wild-type anti-CD56 antibody, which initially showed a narrower separation window relative to the BioLegend standard.

A critical quality challenge for antibody reagents lies in their inherent instability during storage at 4 °C, the most convenient and universally accepted temperature for short-term storage. This temperature allows for the immediate retrieval and subsequent repeated use of antibody reagents without compromising the entire sample. Conventionally, the long-term stability of native antibodies—regardless of their origin (hybridoma screening, phage display libraries, or rational design)—is difficult to predict. However, our *in vitro* evolution platform, which utilizes high-temperature-resistant CHO cells to display antibody variants, effectively addresses this issue by rigorously selecting for those rare clones with inherently stable characteristics (Luo *et al.* 2022). Consequently, even inherently unstable parental antibodies can be matured into highly stable reagents through this platform.

The detailed analysis of the melting temperatures of anti-CD33 and anti-CD56 antibodies using UNcle Protein Stability Analysis Platform revealed the instability of these antibodies across the whole range of temperatures between 25 °C and 100 °C (**Figure 5B** and **5C**). This ability is especially valuable. For example, although the differences of the gross melting temperatures between wild type and improved mutant antibodies are modest and close, 4.4 °C for anti-CD33 and 5.3 °C for anti-CD56 (**Figure 5D**), respectively, the lower melting temperatures of the wild type anti-CD33 antibody over the low temperature range causes much more severe problems in stability than the wild type anti-CD56, indicated by PAGE gel analysis (**Figure 5A**). Thus, antibodies such as this anti-CD33 antibody from domestic antibody manufacturers in China are expected to exhibit significant improvements in stability following application of our *in vitro* evolution protocol.

After *in-vitro* evolution, the anti-CD33 and anti-CD56 variants exhibited elevated T_m values accompanied by reduced aggregation and fragmentation (**Figure 5**). Nevertheless, direct experimental evidence establishing a causal link between increased T_m and diminished cold-induced aggregation and fragmentation remains scarce in published literature. While enhanced conformational rigidity, as reflected by a higher T_m, would be anticipated to limit the abundance of partially-unfolded intermediates responsible for cold-stress-related degradation, T_m characterizes protein unfolding at high temperatures and cannot reliably predict cold-denaturation behavior. Further dissection of this mechanistic connection would therefore greatly reinforce the rationale underlying our stability-oriented antibody-engineering strategy.

It is worth noting that locus-targeted dCas9-AID mutagenesis systems deliver outstanding mutagenic efficiency, achieving drastically higher mutation frequencies within defined antibody gene regions (Hess *et al.* 2016). Rather than inducing random genome-wide mutagenesis, this system recruits AID to predefined loci via dCas9 guidance, thereby concentrating somatic hypermutation exclusively at the gene of interest to boost target mutation rates. By contrast, our platform features several exclusive strengths built upon two core components: an engineered thermotolerant CHO cell line (Luo *et al.* 2022) and a hyperactive mutated AID variant (Luo *et al.* 2020). Our CHO host cells carry a single fixed chromosomal expression cassette that permits seamless insertion of antibody coding sequences and drives robust, stable transgene expression—a prerequisite for AID to generate abundant gene variants. Moreover, the thermoresistant phenotype enables culture at 41 °C: antibody variants with poor structural stability fail to fold correctly and lose antigen-binding capacity under elevated temperature. This screening pressure ensures recovered clones exhibit simultaneous improvements in both cellular detection and thermal stability. Compared with common human cell lines (HEK293, HeLa, K562), CHO cells have a shorter doubling time and far greater tolerance to repeated rounds of FACS sorting. These two characteristics drastically streamline iterative multi-cycle *in vitro* evolution workflows. Industrially, CHO cells represent the dominant workhorse for antibody manufacturing. Since our affinity maturation platform operates entirely within CHO hosts, top mutant clones directly translate to production-scale CHO expression without host cell switching, eliminating compatibility risks introduced by cross-system transfer. Regarding our AID construct, multiple amino acid substitutions were

introduced into the wild-type AID backbone, endowing the enzyme with substantially elevated intrinsic mutagenic activity. In future work, we envision constructing a hybrid platform that synergizes the merits of dCas9-guided locus-specific hypermutation and our thermotolerant CHO cell-based evolution system.

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Compliance with Ethics Guidelines

Conflict of Interest:

Qian Yao, Yun Zhao, Junying Jia, Shu Meng, Xin Zhang, Zhishang Hu, Haiying Hang, Yang Cao, Ruiqi Luo, Yanshu Zhang declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5).

Informed consent was obtained from all patients for being included in the study.

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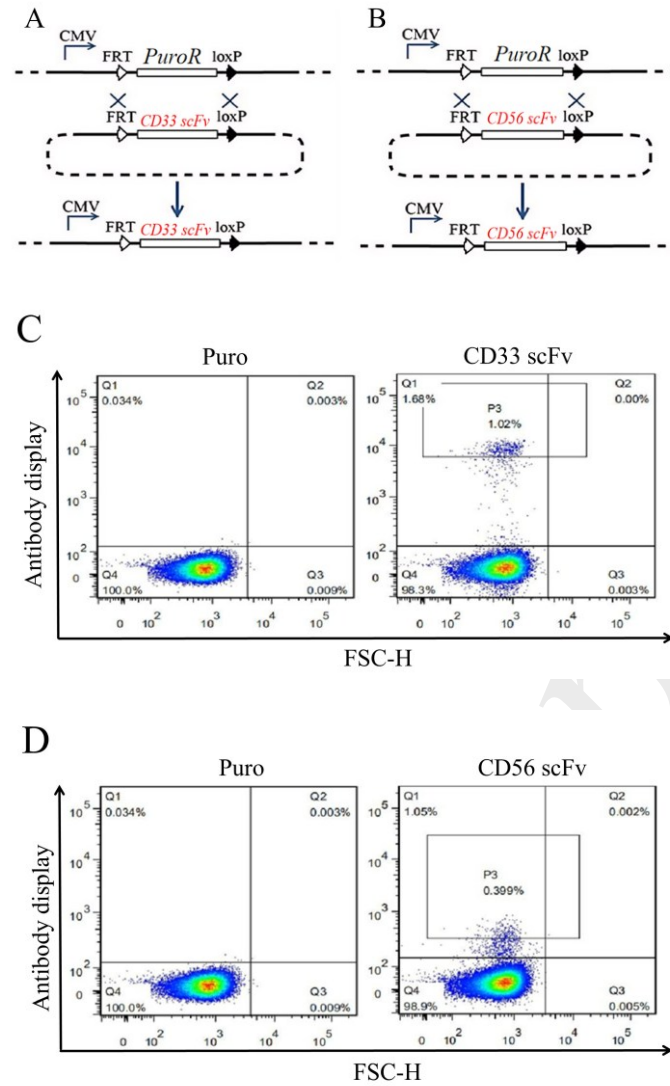


Figure 1. Single strain *anti-CD33* (A) or *anti-CD56* (B) antibody genes were inserted into the gene cassette in CHO cells by replacing *PuroR* gene via recombination. The cells in which an antibody gene was successfully inserted were enriched by flow sorting before the genes were mutated (C) and (D). Puro cells were the CHO cells harboring *Puro* gene in the gene cassette and did not display antibody on the cells surface.

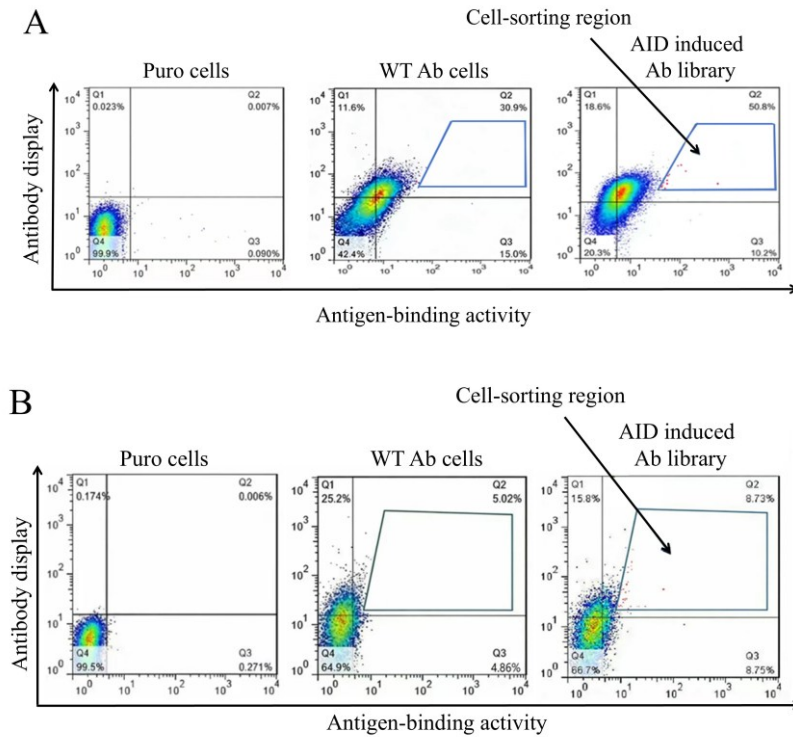


Figure 2. The anti-CD33 (A) and anti-CD56 (B) mutant antibody libraries were constructed as described below. Proliferation of CHO cells expressing anti-CD33 (A) and anti-CD56 (B) was accompanied by diversification of the antibody gene, leading to the establishment of mutant libraries. Cells displaying red dots—predicted to harbor mutant antibodies with both improved affinity and stability—were enriched by flow sorting (AID induced Ab library). Cells harboring wild type anti-CD33 and anti-CD56 (WT Ab cells) did not show such red dots in flow cytometry plots. Puro cells were the CHO cells harboring *Puro* gene in the gene cassette and used as negative control

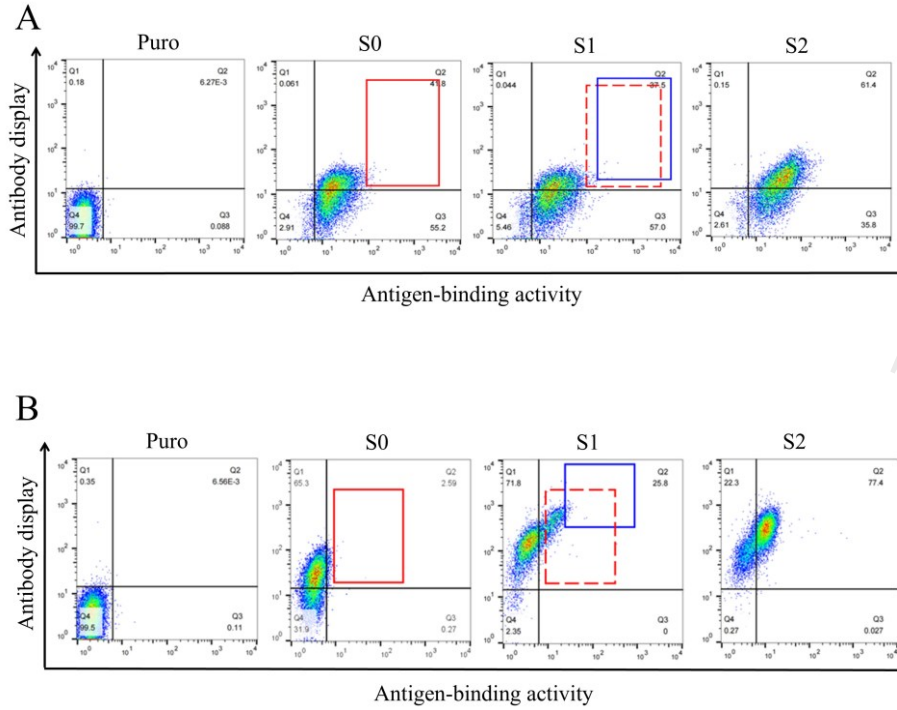


Figure 3. *Anti-CD33* (A) or *anti-CD56* (B) gene was being mutated by AID enzyme in CHO cells while the cells were proliferated forming mutant an *Anti-CD33* (A) or *anti-CD56* (B) gene library (S0 stage). The cells harboring antibodies with improved anti-gen-binding activity as well as display (inside S0 red box) were enriched by flow cytometry sorting. These enriched cells in S0 stage proliferated again and the cells harboring antibodies with further improved anti-gen-binding activity as well as display (inside S1 blue box) were enriched again by flow cytometry sorting. These enriched enriched cells formed a cell population the majority of which had higher antigen-binding activity and antibody display (S2) than those in S0 and S1. Puro cells were the CHO cells harboring *Puro* gene in the gene cassette and used as negative control. WT: wild type

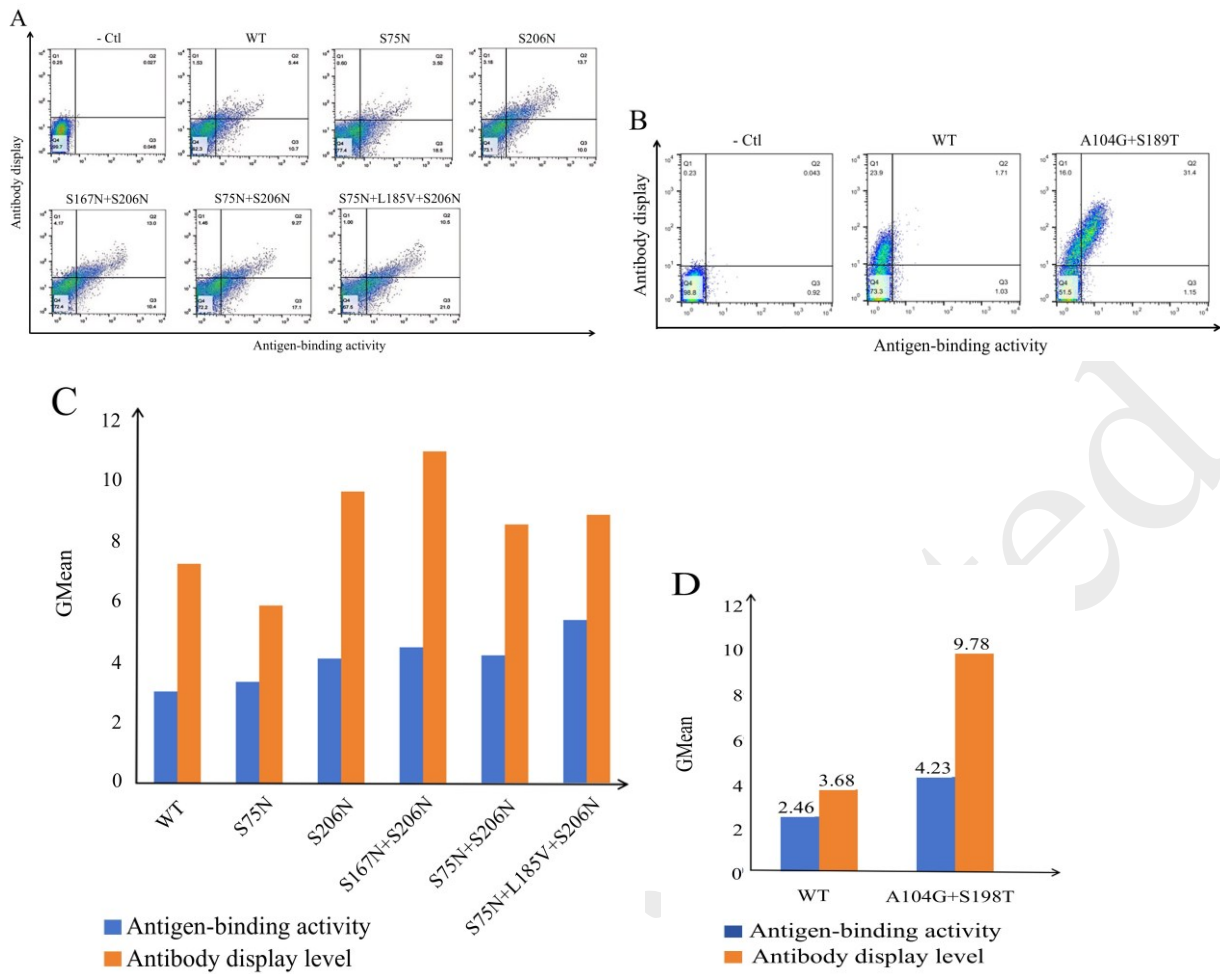


Figure 4. Antigen-binding activities and antibody display levels of the anti-CD33 (A) and anti-CD56 (B) mutant antibodies and the wild type parental antibodies were compared. The geometric means of antigen-binding activities and antibody display levels of the anti-CD33 (C) and anti-CD56 (D) were generated respectively from (A) and (B) using flow cytometric analysis tool. – Ctl: cells harboring *Puro* gene and used as negative control

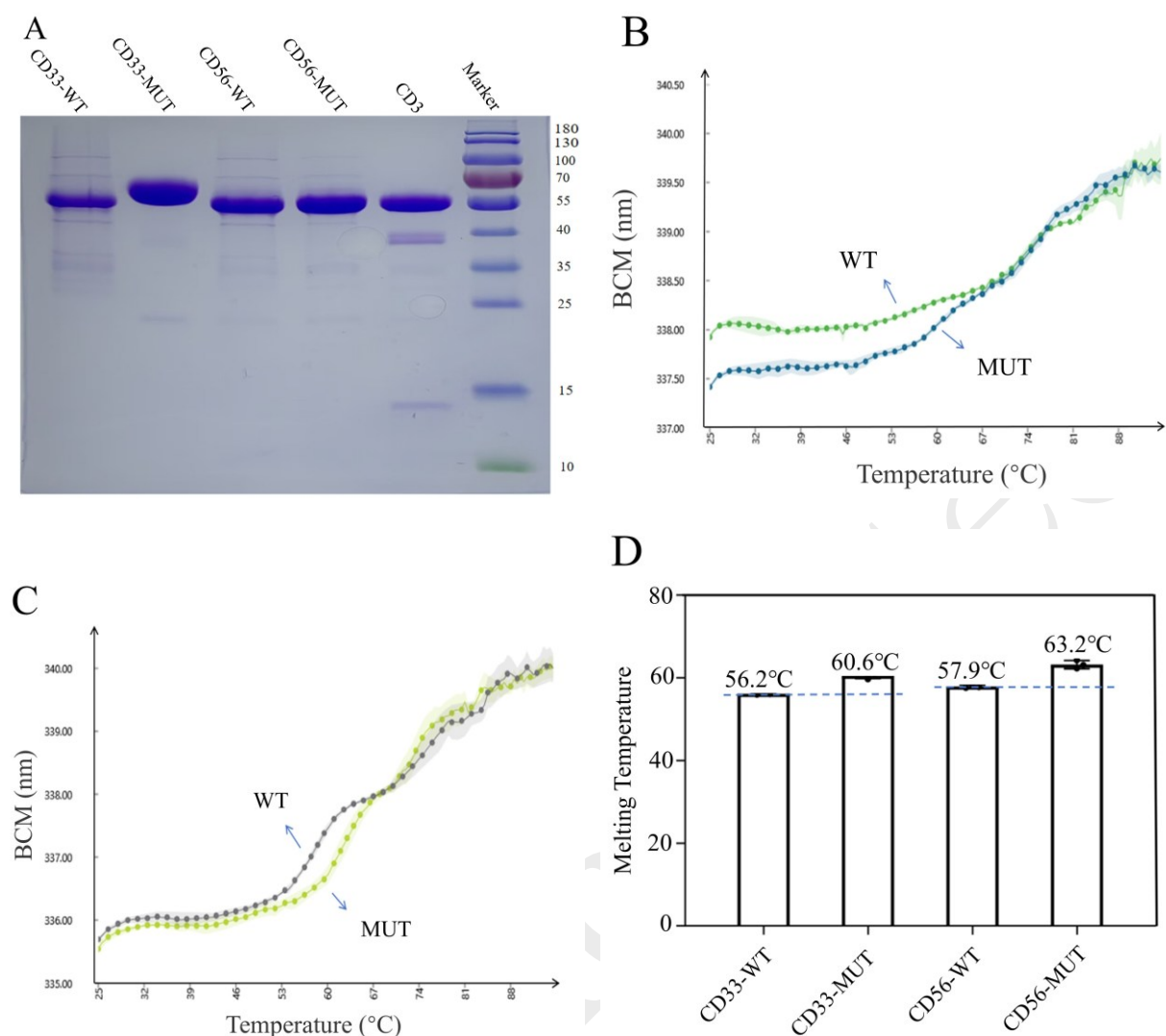


Figure 5. The stabilities of anti-CD33 and anti-CD56 mutant antibodies. The antibodies are in the form of scFv-Fc. An anti-CD3 antibody was used as an additional control (A). Equal amount of the antibody protein samples was loaded on gel. UNcle Protein Stability Analysis Platform was used to measure denaturation and aggregation of the antibody proteins while increasing temperature of buffer (B). BCM (Barycentire Mean) is a comprehensive measurement of protein denaturation and aggregation, negatively associated with protein stability. The melting temperatures of the antibodies were quantitatively compared (C).

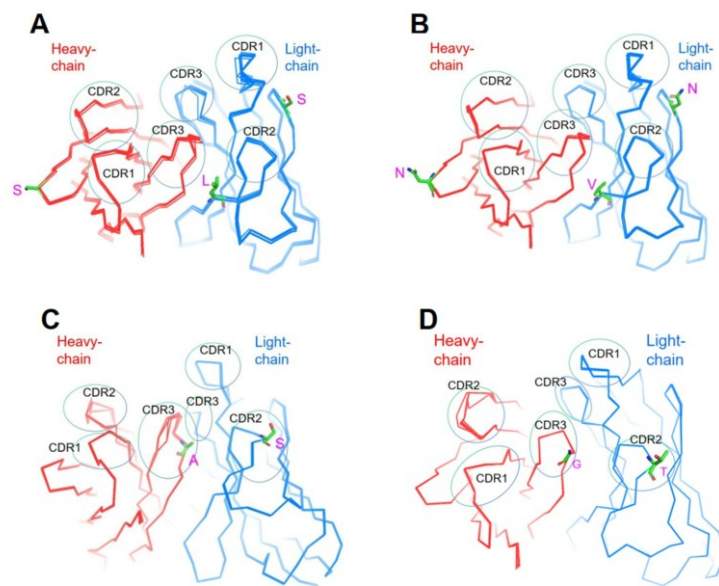


Figure 6. Modeled structures of CD33 and CD56 antibodies. **A** (wild-type) and **B** (mutant) are CD33 antibodies; **C** (wild-type) and **D** (mutant) are CD56 antibodies.

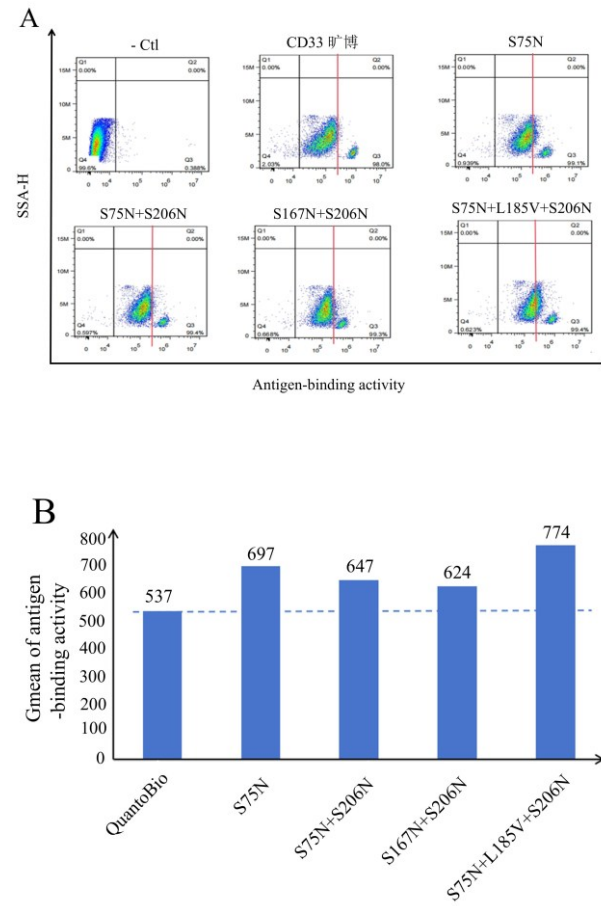


Figure 7. Comparisons on detecting human peripheral leukocytes between the mutant anti-CD33 antibodies and the corresponding commercial one. The flow cytometry plots (A) and the geometric means (B) are presented.

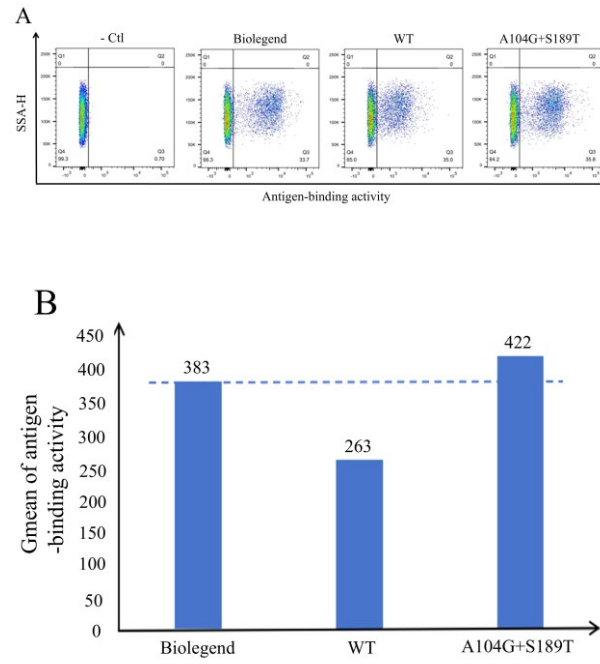


Figure 8. Comparisons on detecting human peripheral leukocytes between the anti-CD56 antibodies and a commercial antibody. The flow cytometry plots (A) and the geometric means (B) are presented.

Table 1 All mutant antibody genes used in this research

Antibody type	Mutation type	Mutant number	%
Anti-CD33	S75N(HC)	17	34
	S206N(LC)	12	24
	S75N+S206N	6	12
	S167N(LCCDR1)+S206N	1	2
	S75N+L185V (LC)+S206N	2	4
Anti-CD56	A104G (HCCDR3) +S189T (LCDR2)	26	52

HC: heavy chain; LC: light chain; LCCDR1: complementarity–determining region 1 of light chain; LCCDR2: complementarity–determining region 2 of light chain; HCCDR3: complementarity–determining region 3 of heavy chain.

Table 2 The geometric means (GMean) of antibody display levels and antigen-binding activities

Anti-CD33 antibody clones	GMean antibody display level	GMean antigen-binding activity
WT	3.03	7.28
S75N	3.37	5.9
S206N	4.14	9.66
S167N+S206N	4.51	11
S75N+S206N	4.25	8.58
S75N+L185V+S206N	5.4	8.89