Rapid discovery of CD28-specific antibodies from rabbits through single B cell cloning on the Beacon system

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ABSTRACT

CD28 is a costimulatory receptor for T cell receptor activation, and CD28 binding antibodies are in clinical development to block or promote T cell activation[1,2]. CD28-specific binding domains are also used in bi- or tri-specific antibody formats to generate T cell engaging antibodies[3]. We set out to generate CD28-specific mAbs from rabbits using a single B cell cloning workflow on the Beacon system. The Beacon system enables rapid antibody discovery using a function-first approach. Antibody-secreting cells (ASCs) are isolated from immunized animals and individually placed into nanowells (pens) of a Beacon chip. Due to the small size of the pens, the secreted antibody concentration reaches µg/ml levels within minutes, and the functional properties of the antibodies can be tested in sequential or multiplexed bead-based assays with a fluorescent readout. Typical assay duration is 30-60 minutes.

Rabbits were immunized with CHOK1 cells stably transfected to carry human CD28 (hCD28) on their surface. At the end of the immunization schedule, memory B cells were isolated from blood using Ficoll separation and magnetic sorting. B cells were cultured in an activation media, and cells were analyzed after 4 and 5 days of activation. After activation, the average cell size increased and cells proliferated roughly 15-fold, with cell numbers unchanged after that. Flow cytometry showed a reduction of surface IgG and a clear increase of intracellular IgG during activation, consistent with a shift from B cells to antibody secreting cells. On day 4 and 5, a total of 32,716 activated B cells were screened on Beacon, first for overall IgG secretion and then for IgG specific for hCD28. 14,889 cells secreted IgG (76%), and 108 cells secreted IgG specific to hCD28 (0.43% of IgG secretors). cDNA from identified hits was individually exported from the chip and submitted for VH/VL sequencing.

Antibody discovery on the Beacon enabled rapid screening of a large number of ASCs and identification of antigen-specific antibodies within 4 days of obtaining PBMCs from immunized rabbits. Of 23 recombinantly-expressed antibodies, most confirmed good binding to CD28 while several showed superior binding and receptor activation compared to a reference antibody from a large pharmaceutical company.

MATERIALS AND METHODS

Immunization: A New Zealand White rabbit was subcutaneously immunized at various time points with 100 million CHO-K1 cells expressing human CD28 (hCD28) on their surface.
Titer testing: Serum titer was tested by ELISA and flow cytometry. For ELISA, hCD28 ECD was coated onto ELISA plates. For cytometry, HEK293 cells expressing hCD28 and parental cells were incubated with serum, followed with a fluorescently labelled anti-rabbit IgG.
PBMC thawing, memory B cell purification and activation: PBMC thawing, memory B cell isolation and activation followed the protocol provided by Bruker. Briefly, a biotinylated antibody specific for IgG^+ memory B cells was added, the cells washed, and anti-biotin microbeads were added. Cells were applied to two consecutive magnetic column purifications. Resulting memory B cells were resuspended in an activation and stimulation medium, seeded at 5,000 cells/well in 96-well plates, and incubated for up to 6 days at 37°C and 5% CO_2.
Cell size, viability and flow cytometric analyses: Before and during activation, cells were analyzed for counts, viability and size on a Luna cell counter. For flow cytometric analyses, cells were incubated with fluorescently-labelled antibodies for surface markers (IgG, rabbit plasma cell marker). After washing cells were fixed and permeabilized and labelled for intracellular Igκ.
Plasma Cell (PC) isolation: PCs were isolated using a proprietary magnetic isolation protocol and directly used on Beacon.
On Beacon antibody discovery: ASCs and PCs were penned onto an OptoSelect 20K chip. A bead-based antigen binding assay was performed using capture beads (Spherotech) and hCD28 protein labelled with Alexa Fluor-488 as detection reagent. Similarly, a bead-based IgG secretion assay was performed using IgG capture beads and an anti-IgG H+L Alexa Fluor-647 as a detection reagent.
Antibody characterization: Recombinant antibodies were tested for binding and functional activity using standard *in vitro* immunological methods as described in the figure legends.

FIG.1 RABBIT IMMUNIZATION, TITER, CELL ISOLATION AND CULTURING

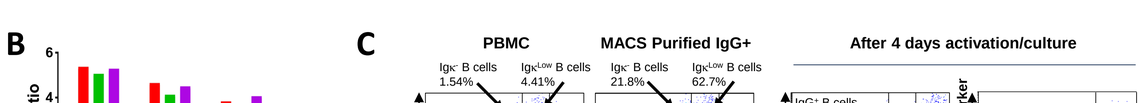
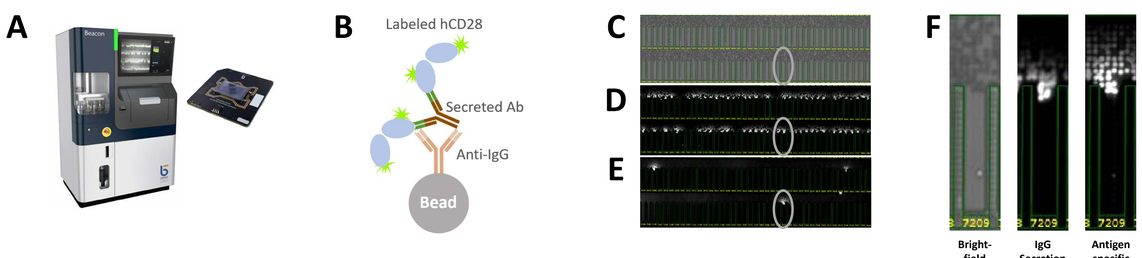


Figure 1. Isolation of rabbit ASCs/PCs from memory B cells. (A) A rabbit was immunized with hCD28-CHOK1 cells, test bleeds (TB) were collected and screened for binding to **(B)** cell-surface hCD28 by flow cytometry (MFI ratio of transfected vs. parental cells is shown) and by ELISA (not shown). **(C)** Characterization of PBMC and memory B cells. PBMC contained ~6% memory B cells (IgG^+), with roughly ¾ $Igκ^{Low}$ B cells, and ¼ $Igκ^-$ B cells. After purification, IgG+ memory B cell fraction increased to ~85%. After 4-5 days of activation, $Igκ^{High}$ ASCs increased from 0.3% to 90% and cells became surface IgG^{Low}. The remaining $icIgκ^-$ cells, likely lambda PCs, also became surface IgG^{Low}. During our efforts to identify rabbit PCs, we identified one surface marker that gives a high correlation with ASCs/PCs (as identified by $icIgκ^{High}$).

FIG.2 ANTIBODY DISCOVERY ON BEACON



G	Single B cells screened	IgG secretors	Secretion rate	Anti-hCD28 hits	Specific rate
B cell, Day 4	16,805	12,839	76%	54	0.42%
B cell, Day 5	15,911	12,050	76%	54	0.45%
Total	32,716	24,889	76%	108	0.43%

Figure 2. Antibody discovery on Beacon. (A) Antibody discovery was conducted in nanofluidic chips on Beacon with cells from Day 4 and Day 5 of activation. **(B)** Bead-based assays with fluorescent readouts were used to identify total IgG secretion or IgG specific for hCD28. A section of the 20k microfluidic chip showing 82 pens is shown in **(C)** bright-field, **(D)** FITC-channel, and **(E)** Cy5-channel, with pen 7209 circled. Individual cells were loaded in pens and assay beads in the channels. The appearance of a fluorescent "bloom" in the FITC- and Cy5-channel identified IgG secretors and specific hits, respectively. **(F)** As an example, magnification of Pen 7209 shows that it contains a single plasma cell that secretes IgG specific for hCD28. **(G)** Overview table showing numbers of screened cells and identified hits.

FIG.3 SEQUENCE DIVERSITY

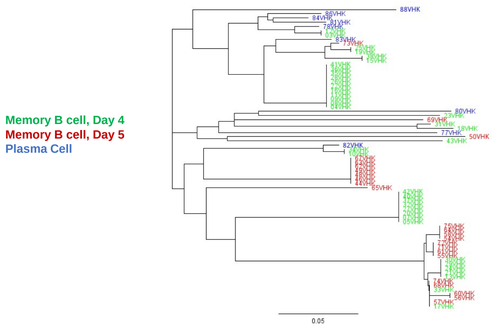


Figure 3. Paired VH/VL sequence diversity. Antibody sequences were derived from three runs, including Day 4 (green), Day 5 (red) after memory B cell activation and from splenic Plasma Cells (blue). Due to sampling of only 1 or 2 culture wells, of 70 VH/VL paired sequences, 28 were considered unique. Sampling more wells increases overall sequence diversity. 23 unique pairs were selected for re-expression and were carried forward for subsequent *in vitro* characterization. For Plasma Cell VH/VL paired sequences, 9 of 9 were considered unique.

FIG.4 CHARACTERIZATION BY ELISA AND FLOW CYTOMETRY

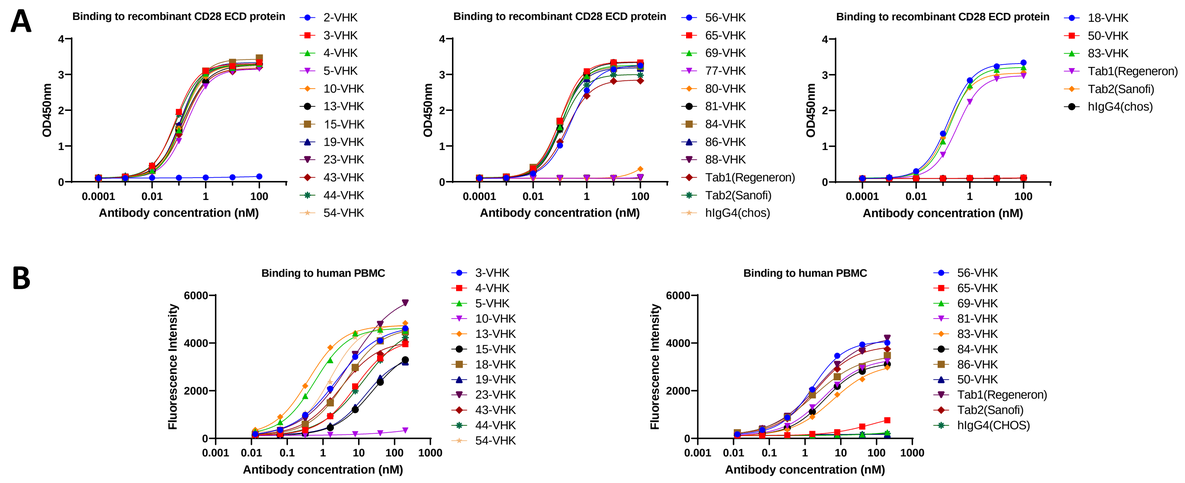


Figure 4. Binding to receptor and cells. (A) Protein-based ELISA capture assay against recombinant human CD28 ECD His tag protein. Five of 23 expressed mAbs had EC50s below the benchmarks. **(B)** Binding to donor PBMC where amount bound was detected using a fluorescently labeled secondary antibody. Five mAbs had EC50s below the benchmarks.

FIG.5 CELL-BASED RECEPTOR BLOCKING ASSAY AGAINST B7.1 AND B7.2

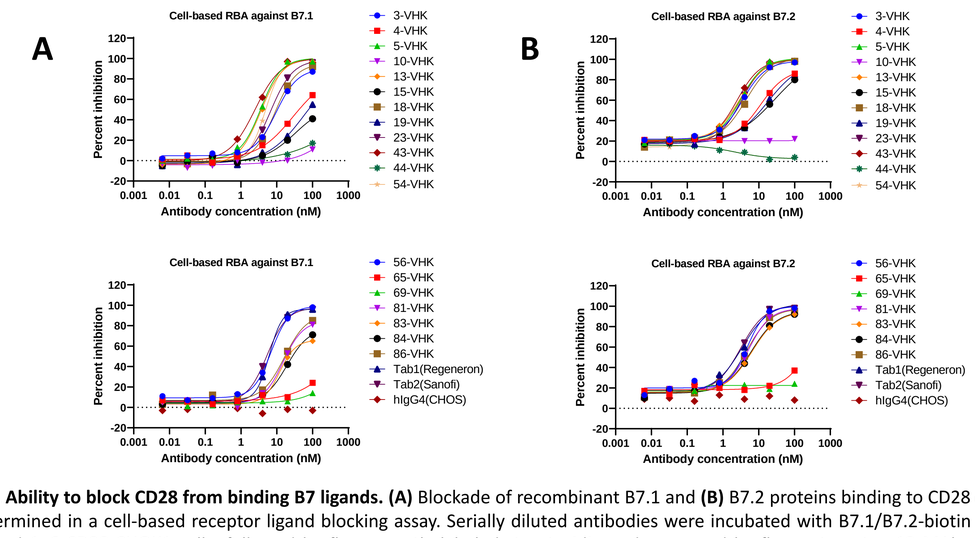


Figure 5. Ability to block CD28 from binding B7 ligands. (A) Blockade of recombinant B7.1 and **(B)** B7.2 proteins binding to CD28 on CHOK1 cells was determined in a cell-based receptor ligand blocking assay. Serially diluted antibodies were incubated with B7.1/B7.2-biotin proteins (at 15 µg/ml) and 1e6 CD28-CHOK1 cells, followed by fluorescently labeled streptavidin and measured by flow cytometry. 12 MAbs could block B7 ligands, with four having IC50s below the benchmarks for B7.1 and three for B7.2.

FIG.6 T CELL RECEPTOR CORECEPTOR ACTIVITY ASSAY

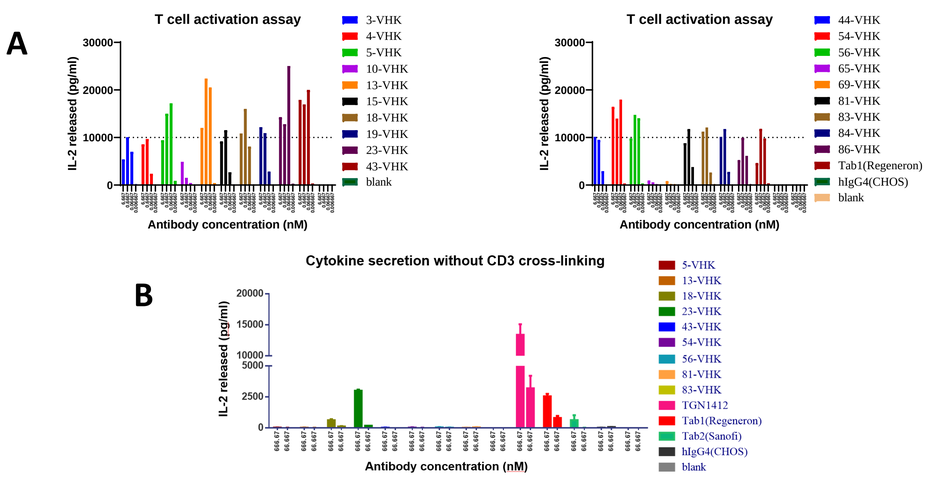


Figure 6. Primary T cell activation assay. (A) To examine whether purified anti-CD28 antibodies can contribute to full T cell activation, donor PBMC were incubated with 1 µg/ml plate-bound anti-CD3ε antibody (OKT-3) and 2.2 µg/ml plate-bound cross-linking antibody (anti-human Fc) and candidate CD28 antibodies. After 48 hours, IL-2 within the culture supernatant was measured by ELISA. Six mAbs induced higher IL-2 secretion than the benchmark. **(B)** Three mAbs could induce IL-2 secretion without CD3 crosslinking, suggesting super-agonist activity.

FIG.7 BINDING AFFINITY VIA BIACORE

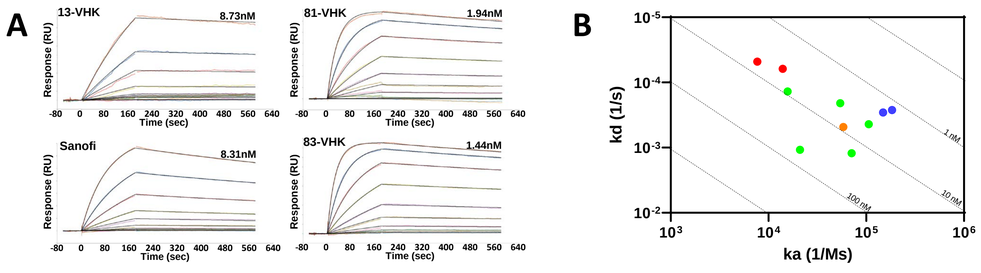


Figure 7. Receptor binding affinity. (A) Four representative surface plasmon resonance sensograms. **(B)** Isoaffinity plot summarizing affinity data for the 10 mAbs tested. mAbs are from memory cells (red/green), splenic Plasma Cells (blue), and a reference mAb (orange).

CONCLUSIONS

Because rabbits can generate antibodies against epitopes conserved between humans and mice, have higher affinities, have CDR3 lengths more similar to humans and have increased antibody diversity[4], there is a high interest in discovering antibodies from these animals. Rabbits are frequently used to generate polyclonal antisera, however, due to the lack of efficient methods they are rarely used for monoclonal antibody discovery.

Here we describe how the Beacon system enabled us to discover anti-human CD28 monoclonal antibodies from rabbits in an extremely short time span. The discovery itself was completed in 4 to 5 days. 19 recombinantly-expressed antibodies performed well in standard immunological assays, with ~1/4 of antibodies tested showing greater binding and IL-2 induction than reference antibodies. Interestingly, the binding affinity profiles of the two antibodies derived from natural Plasma Cells were greater than all antibodies tested from memory B cells (and the reference). This B cell cloning method proved to be robust and efficient in our hands, leading to a sizeable number of anti-CD28 monoclonal antibodies from a single rabbit that show excellent receptor binding and activation characteristics.

REFERENCES

[1] Nat Rev Drug Discov. 2020 Dec; 19(12):860-883. [2] Diseases. 2018 May 19;6(2):41. [3] Nature 2022 Mar; 603(7900):328-334. [4] Exp Mol Med 2017 Mar 24; 49(3):e305.