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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 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 placed individually 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. Serum samples showed clear, yet low specific titers for hCD28. 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. During the activation period, the average cell size increased and cells proliferated roughly 15-fold until day 5, 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. cDNA generation and export happened on the day of the Beacon run, and cDNA amplification added another day to the workflow. The method was robust and substantially reduced the hands-on time for rabbit antibody discovery.

MATERIALS AND METHODS

Immunization: A New Zealand White rabbit was subcutaneously immunized with 100 million CHO-K1 cells expressing hCD28 on their surface. The first 4 immunizations were given on days 0, 14, 28 and 66 with Mn^{2+} jelly as adjuvant. The final boost was given on day 87 without adjuvant. The rabbit was sacrificed on day 90 and blood, spleen and BM were harvested.

Titer testing: Serum titer to hCD28 was tested by ELISA and flow cytometry. For ELISA, hCD28 ECD was coated onto an ELISA plate, washed and hydrophobic interaction sites were blocked with BSA. Serum dilutions were added, after an incubation period the plates were washed well, anti-rabbit IgG HRP-conjugate was added, and after another wash step TMB was added. The color reaction was stopped with acid and the plates were read at 450 nm. For flow cytometry, HEK293 cells expressing hCD28 and untransfected HEK293 cells were washed, incubated with dilutions of rabbit serum, washed, and incubated with a fluorescently labelled anti-rabbit IgG. After washing cells were analyzed by flow cytometry.

PBMC isolation and freezing: Blood was diluted 2-fold with PBS, and separated on Ficoll-Paque by centrifugation for 30 min at 400 g. The PBMC layer was carefully recovered, the cells were washed with PBS, and resuspended to 100 million cells per ml in a proprietary freezing media. 1 ml aliquots were filled into cryovials and frozen in cryo-preservation freezing racks at -80°C, before being transferred to the gas phase of liquid nitrogen storage tank on the next day.

PBMC thawing, memory B-cell purification and activation: PBMC thawing, memory B-cell isolation and activation followed the protocol provided by PhenomeX. Briefly, PBMCs were thawed, washed, and resuspended in buffer containing 5% normal mouse serum. A biotinylated antibody specific for IgG^+ memory B-cells was allowed to bind, the cells were washed, and anti-biotin microbeads were added. After washing, cells were applied to a magnetic column, washed, eluted, and the purification process was repeated on a 2nd column. Resulting memory B-cells were resuspended in an activation and stimulation medium, seeded into 96 well plates at 5,000 cells per well, 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 on days 0, 3, 4, 5 and 6. Cell count, viability and size was determined on a Luna cell counter. For flow cytometric analyses, cells were washed, IgG receptors were blocked (where applicable), and cells were incubated with fluorescently-labelled antibodies for surface markers (IgG, rabbit plasma cell marker). After washing and blocking of surface Igkappa (Igκ), cells were fixed and permeabilized and labelled for intracellular Igκ. Cells were washed prior to cytometry.

On Beacon antibody discovery: In vitro differentiated ASCs from an immunized rabbit were penned onto an OptoSelect 20K chip. The 20K chip contains approximately 20,000 individual nanowells (pens) to capture single cells. A bead-based antigen binding assay was performed using anti-rabbit IgG beads (Spherotech) as capture beads and hCD28 protein fluorescently labelled with Alexa Fluor 488 as detection reagent. Similarly, a bead-based IgG secretion assay was performed using anti-rabbit IgG capture beads and anti-rabbit IgG H+L fluorescently labelled with Alexa Fluor 647 as a detection reagent.

cDNA generation and QC: mRNA capture beads were added to pens containing selected ASCs. Lysis buffer and reagents for reverse transcription were flushed into the chip. After lysis, the reverse transcription process was completed on chip at 40°C after approximately 3-4 hours. cDNA capture beads were sequentially exported to 96-well plates and total cellular cDNA was amplified using primers specific to sequences annealed during the reverse transcription process. The cDNA was quantified using a Nanodrop instrument and quality was assessed using a Labchip instrument (Perkin Elmer).

FIG.1 RABBIT IMMUNIZATION AND TITER

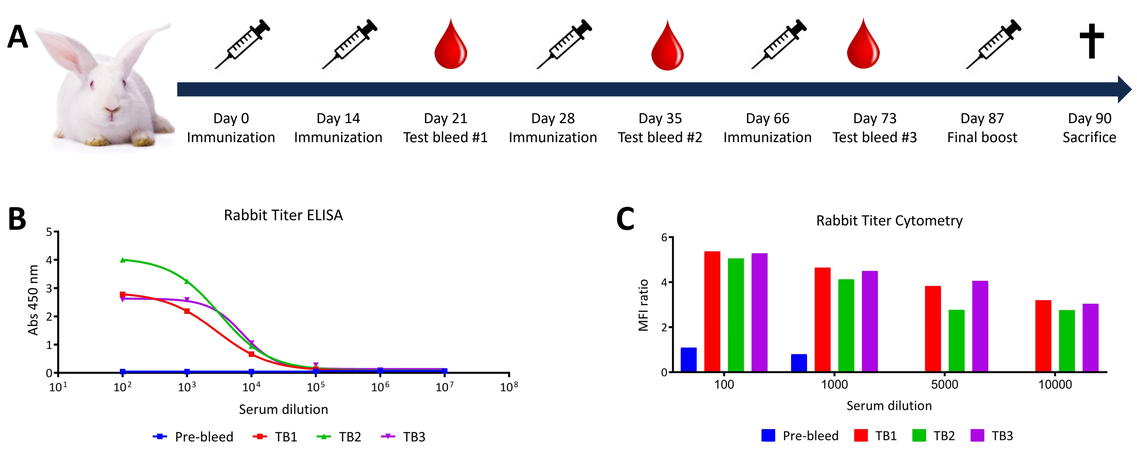


Figure 1. Immunization schedule and titer determination. (A) A New Zealand White rabbit was immunized with human CD28-CHOK1 cells, test bleeds (TB) were collected at regular intervals and screened for binding to **(B)** hCD28-ECD by ELISA and **(C)** cell-surface hCD28 by flow cytometry (MFI ratio of transfected vs. parental cells is shown). The titer does not show substantial increases after the first test-bleed (TB1), indicating that by the third boost a plateau was reached. The hCD28-specific titer is in line with expectations for cell-based immunization.

FIG.2 MEMORY B-CELL ISOLATION

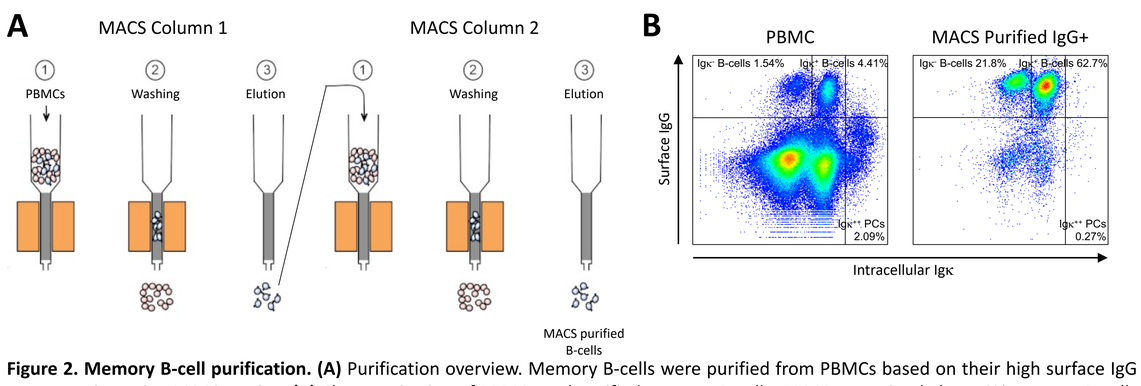


Figure 2. Memory B-cell purification. (A) Purification overview. Memory B-cells were purified from PBMCs based on their high surface IgG concentration using MACS sorting. **(B)** Characterization of PBMCs and purified memory B-cells. PBMCs contained about 6% memory B-cells (IgG^+), with roughly ¾ $Igκ^+$ B-ells, and ¼ $Igκ^-$ B-cells. After purification the total memory B-cell fraction increased to about 85%.

FIG.3 MEMORY B-CELL ACTIVATION

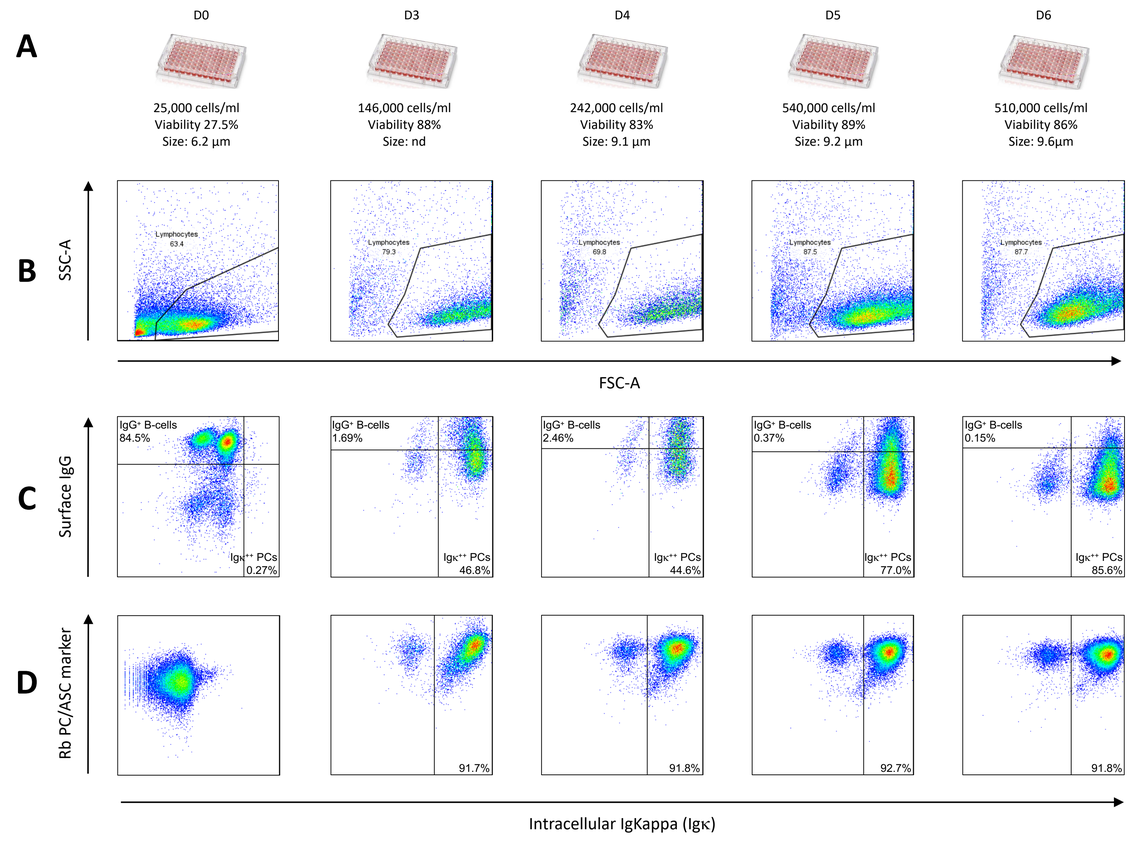
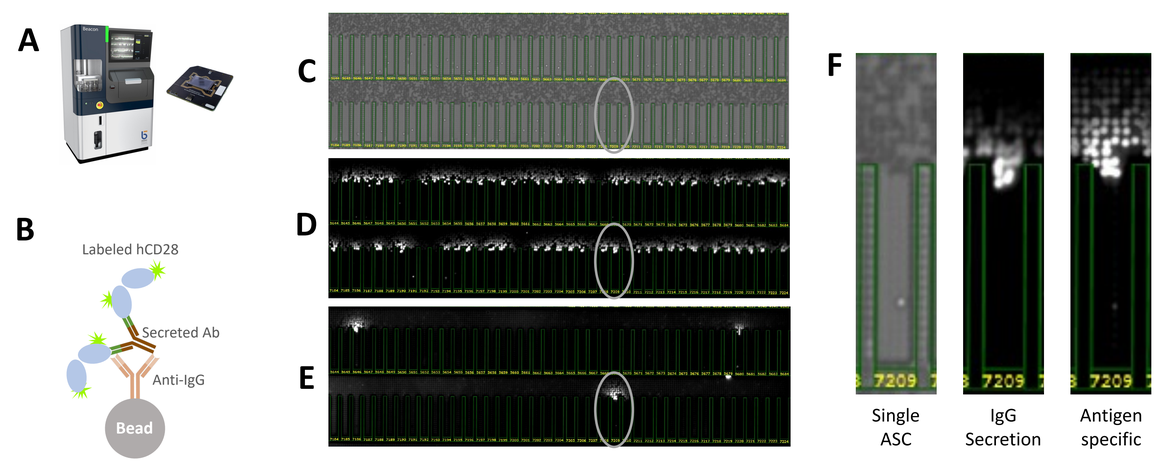


Figure 3. Memory B-cell activation. (A) Purified memory B-cells were cultured over a 6 day period, and proliferation as well as cell size increase indicate successful B-cell activation. Cells reach their highest cell number and viability on day 5, with a decrease to day 6 visible in both parameters. **(B)** Blasting cells were characterized by flow cytometry on days 3, 4, 5 and 6. Forward scatter shows the size increase. **(C)** Robust and specific surface markers for rabbit ASCs (or plasma cells) have not been described in literature. Flow cytometric analysis of surface IgG (sIgG) and intracellular Igκ (icIgκ) however allows identification of B-cells ($sIgG^{++}$) and Igkappa-secreting PCs ($sIgG^{lo}$, $icIgκ^{++}$). During activation of the memory B-cells, the kappa-positive PC concentration increased from 0.2% to over 80%. The remaining $sIgG^{lo}$ $icIgκ^-$ cells are likely lambda PCs. **(D)** During our efforts to unambiguously identify rabbit ASCs (or PCs) we identified one surface marker that gives a high correlation with ASCs/PCs (as identified by $icIgκ^{++}$).

FIG.4 ANTIBODY DISCOVERY ON BEACON



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	Single B-cells screened	IgG secretors	Secretion rate	Anti-hCD28 hits	Specific rate
1st run	16,805	12,839	76%	54	0.42%
2nd run	15,911	12,050	76%	54	0.45%
Total	32,716	24,889	76%	108	0.43%

Figure 4. 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 cells secreting total IgG or IgG specific for hCD28. **(C-E)** A section of the 20k microfluidic chip showing 82 pens is shown **(C)** in bright-field, **(D)** in the FITC-channel, **(E)** and in the Cy5-channel, with pen 7209 circled. (C) Individual cells were loaded into the pens and assay beads were loaded into the channel. The appearance of a fluorescent "bloom" in the (D) FITC-channel and in the (E) 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.5 cDNA GENERATION AND QC

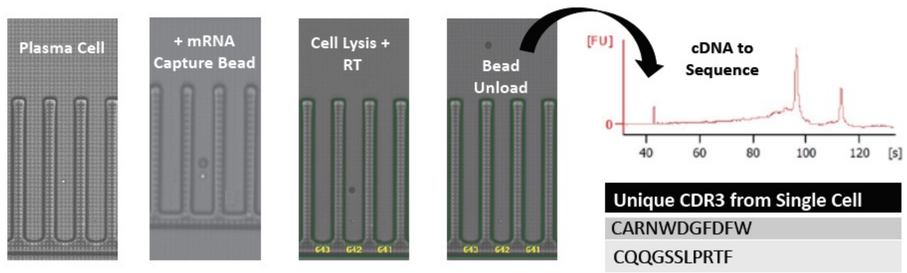

Figure5. cDNA generation. Cells were lysed on chip, mRNA was captured onto poly-T beads and cDNA was generated. Beads were exported from the chip, total cellular cDNA was amplified, amount and size distribution was determined, and samples submitted for VH/VL sequencing

CONCLUSIONS

Because rabbits can generate antibodies against epitopes conserved between humans and mice, have higher affinities, have CDR3 lengths 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, cDNA generation and QC took another 2 days. The major determinant of the discovery timeline is the duration of the rabbit immunization. The method proved to be robust and efficient in our hands, leading to a sizeable number of anti-CD28 monoclonal antibodies from a single rabbit.

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.