MABS
2021, VOL. 13, NO. 1, e1978130 (11 pages)
https://doi.org/10.1080/19420862.2021.1978130

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REPORT

Versatile and rapid microfluidics-assisted antibody discovery

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ABSTRACT

Recent years have seen unparalleled development of microfluidic applications for antibody discovery in both academic and pharmaceutical research. Microfluidics can support native chain-paired library generation as well as direct screening of antibody secreting cells obtained by rodent immunization or from the human peripheral blood. While broad diversities of neutralizing antibodies against infectious diseases such as HIV, Ebola, or COVID-19 have been identified from convalescent individuals, microfluidics can expedite therapeutic antibody discovery for cancer or immunological disease indications. In this study, a commercially available microfluidic device, Cyto-Mine, was used for the rapid identification of natively paired antibodies from rodents or human donors screened for specific binding to recombinant antigens, for direct screening with cells expressing the target of interest, and, to our knowledge for the first time, for direct broad functional IgG antibody screening in droplets. The process time from cell preparation to confirmed recombinant antibodies was four weeks. Application of this or similar microfluidic devices and methodologies can accelerate and enhance pharmaceutical antibody hit discovery.

ARTICLE HISTORY
Received 11 May 2021
Revised 2 September 2021
Accepted 3 September 2021

KEYWORDS
Microfluidics; ASCs; antibodies; functional screening; Cyto-Mine

Introduction

The high numbers of biotherapeutics in late clinical trials and approval rates for cancer and autoimmune diseases indicate their remarkable safety and specificity, as well as a powerful, matured development pipeline. Hit discovery for these antibodies was mainly achieved by B-cell cloning[1,2] and hybridoma technology, or from complex variant libraries via phage,[3] yeast,[4] and mammalian display,[5] as recently reviewed by Ministro *et al.*[6] While these methods are streamlined and successful, drawbacks include the need for tedious subcloning of surface-displayed hits into soluble expression formats and loss of diversity during hybridoma generation, often limiting the throughput of screenings, such as for membrane protein binders, and broad function first screens.[7]

Recent years have seen unparalleled development of microfluidic technologies for a plethora of applications. The ability to compartmentalize cells within picoliter droplets revolutionized the study of single cells at the genomic, transcriptomic, proteomic, or metabolomic level,[8] enabled personalized cancer therapy,[9] and included functional phenotyping.[10,11] Microfluidics for antibody discovery originated in academic laboratories and are now a routine antibody discovery platform in pharmaceutical research and development.[12–14] Antibodies secreted from single cells are interrogated after compartmentalization in droplets,[15]nanostructures such as nanopens,[16] or microcapillaries.[17–19] Most antibody secreting cells are plasma cells obtained from rodent immunization,[20] but also differentiated memory B cells, plasma blasts from human peripheral blood,[21,22] or secretion libraries[23] are applied. Antibodies screened by microfluidics retain their native chain pairing, which can be assessed by next-generation sequencing (NGS),[24] used for direct antibody subcloning or subsequent library generation.[25] Compatible with the high throughput of microfluidic setups, mostly fluorescence-based methods are applied for real-time adjustable mining of large diversities. Methodologies range from selection for binding on the recombinant target or cells[26] to internalization or functional screening using reporter gene target cells,[23,27,28] as exemplified by the recent work from Gérard *et al.*[20] While broad diversities of neutralizing antibodies against infectious diseases such as HIV, Ebola, or COVID-19 have been identified from convalescent individuals,[22,29,30] microfluidics can also yield tools[16] as well as therapeutic antibodies targeting cancer or immunological diseases.[31,32]

Several companies offer custom and customizable microfluidic chips for commercial and academic antibody screening campaigns.[12,20,30] In addition, stand-alone devices have recently been made commercially available,[16,23,33] further driving development and diversification of microfluidic methodologies for therapeutic antibody discovery.

Here, we exemplify the broad application range of a commercially available microfluidic device, Cyto-Mine,[33] that can be used for the rapid identification of antibodies from rodents or human donors screened for secretion, specific binding to recombinant antigens or antigen-positive cells, and directly for function in full IgG format. Up to 2 million picoliter-volume droplets containing up to 1 million single antibody-secreting cells

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Supplemental data for this article can be accessed on the publisher's website.

(ASCs) can be screened for envisioned activity as fast as 250 droplets per second. The full process time from cell preparation to confirmed recombinant antibodies was four weeks.

Results

Cyto-Mine workflow

All data presented herein were recorded using the automated Cyto-Mine workflow (Figure 1) for droplet-based high throughput antibody screening with premanufactured cartridge design and the flexibility to apply one of the several assay principles and readouts (Ref. 20 and Figure S1). Generally, up to two million 450 pL droplets were generated from 1 mL input volume containing up to 1 million ASCs, and practically a maximum of 1.944 million droplets could be screened carrying Poisson-distributed ~720,000 ASCs. Although not the focus of this study, microfluidics can be used for rapid sorting of high antibody secreting monoclones. Förster resonance energy transfer (FRET)-based IgG detection was used for successful sorting of ASCs from immunized mice, peripheral blood of vaccinated humans, and hybridoma mixtures, exemplifying the applicability for identification of high secreting clones from diverse origins (Figure S2). Following sorting into a dispensing chamber, single droplet dispensing in 96-well plates filled with lysis buffer for cell lysis, cDNA synthesis, full antibody gene PCR (polymerase chain reaction) recovery, and subcloning allowed rapid and robust generation of recombinant cognate-paired antibody candidates produced for confirmatory secondary screens. Sorting efficiency and overall hit rates varied depending on the input diversity and readout complexity; exemplary statistics are presented in Table 1.

Mining human and murine diversities for target-specific antibodies

In addition to mining neutralizing antibodies against infectious diseases directly from convalescent patients, microfluidics may enable direct cancer patient-derived antibody discovery in the near future. As a surrogate exemplifying the capacity for human diversity mining, high throughput screening of plasma blasts, enriched from the peripheral blood of a tetanus toxoid (TT) vaccinated donor, successfully yielded a panel of 44 sequence diverse and high affinity fully human TT antibodies (Figure 2; Table 1; and supplementary Figures S3, S4, and Table S2). The 44 primary hits from 439 droplets dispensed represent a hit rate of 10%.

At present, microfluidics is mostly used to screen immune diversities generated by immunization of transgenic or wild-type rodents, often mice. We thus included a campaign interrogating enriched plasma cells of wild-type mice immunized with a cancer-related antigen (termed herein "CRA"). Of 635 droplets dispensed, 98 full antibody genes could be identified, subcloned, and produced. Thirty binders were confirmed as target-specific antibodies (Table 1, Figure S4). High affinity could be confirmed for selected reproduced clones (Figure S4C), while sequence evaluation indicated broad diversity (Figure S4). Optimization at multiple steps, including ASC enrichment from selected organs of single animals, is warranted and discussed below.

For both target-specific screening campaigns, fresh or frozen, liquid nitrogen-stored and re-thawed ASCs could be used successfully (Table 1). Storage of up to 10 months in liquid nitrogen followed by 5 days at −80°C, mimicking transport on dry ice, did not significantly alter the viability or secretion capacity of ASCs (Figure S2).

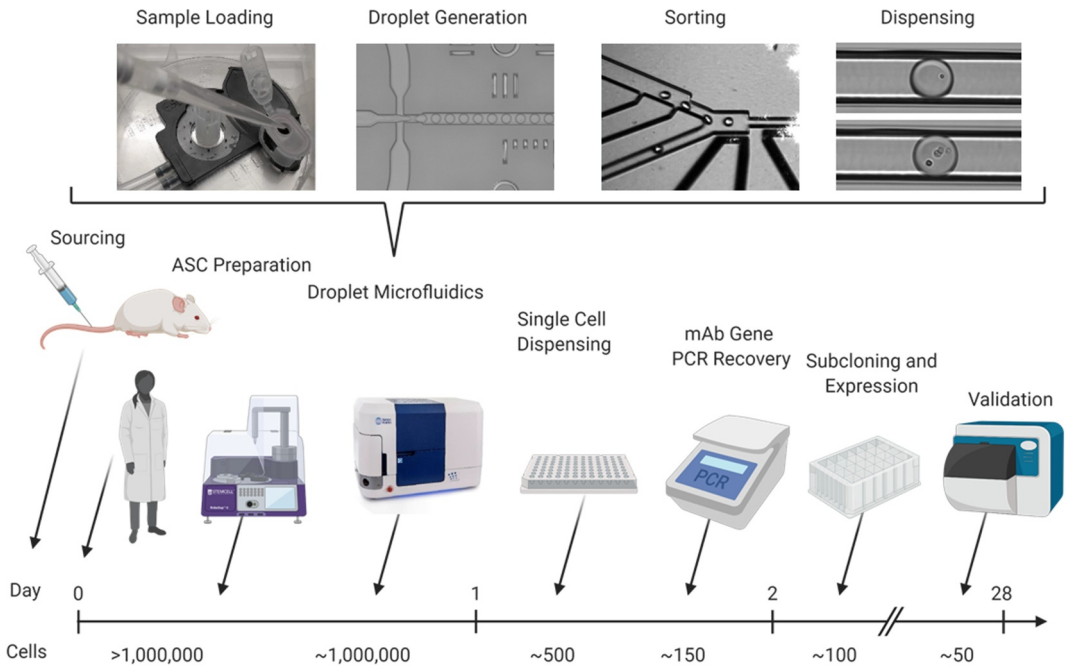

Figure 1. The process of Cyto-Mine antibody hit discovery. Antibody-secreting cells (ASC) can be obtained from sources such as previously immunized rodents or vaccinated human donors. On day 1, cell purification is followed by a droplet microfluidics run consisting of sample loading, droplet generation, sorting for selectivity or function, and often single well dispensing. Subsequently, antibody gene recovery and subcloning, expression, and validation of envisioned antibody properties such as specific binding of cellular function are completed within 4–6 weeks. Cell numbers given for each step reflect typical values that can vary significantly depending on the quality of diversities applied and the stringency and nature of the selection method.

Table 1. Statistics for two Cyto-Mine antibody discovery campaigns. Magnetic-activated cell sorting (MACS) output in the TT campaign represents CD38 + enriched plasma blasts obtained from 297 mL of one healthy vaccinated donor's peripheral blood, whereas CRA campaign MACS output originates from pooled bone marrow, lymph node, and spleen samples of two immunized wild-type mice enriched for CD138+ plasma cells. Of 1050 and 1354 CRA-specific droplets dispensed, 635 and 96 were analyzed and yielded 98 and 33 full monoclonal antibody (mAb) gene PCR amplicons, respectively. 33 of 2 months campaign were not further processed. n.d., not determined.

Step/Antigen	TT		CRA	
ASC source	Freshly prepared	5 days Freeze/thaw	Freshly prepared	2 months Freeze/thaw
Output MACS	5,230,000		6,520,000	
Viability (%)	98	99	95	92
Percent ASCs (%)	n.d.	n.d.	11	9
# Droplets/cells	$2*10^6$/ $2*10^6$	$1*10^6$/$1*10^6$	$2*10^6$/ $1*10^6$	$2*10^6$/$1*10^6$
Sorted	823	221	~2300	~3000
Dispensed	316	117	635	96
Full mAb PCR recovery	83	47	98	33
# mAbs produced	72		92	n.d.
# Target-specific mAbs	44		30	n.d.

Mining for cellular binding omitting recombinant target protein

Mining antibody diversities for cellular binding in droplet-based microfluidic approaches requires co-encapsulation of ASCs with target cells driven by a Poisson distribution. Use of an ASC-to-target cell mixture of 1 to 6 million in 2 million droplets allowed for co-encapsulation and screening of 256,000 ASCs. In a proof-of-concept experiment, a human EGFR (epidermal growth factor receptor)-specific hybridoma mAb108 was spiked 1:100 into irrelevant anti-human CD3 antibody OKT3, and we sought enrichment for cellular binding to co-encapsulated EGFR-positive A431 cells. The portion of mAb108 culture that secreted antibody was measured via enzyme-linked-immuno-spot (ELISpot) to be approximately 30%, yielding 0.3% mAb108 ASCs. Gating for ASC-containing droplets to reduce false-positive background events was enabled by tandem-dye-labeled pan-B-cell detection (488 nm excitation with red channel maximum fluorescence detection), along with the cellular binding detected via anti-Fc -488 and measured via green channel maximum fluorescence signals (Figure 3a–c; Figure S1). Subsequently, dispensing while gating for green maximum vs average was applied as recently described[20] (Figure 3d). About 3,500 droplets were sorted, and of 288 dispensed droplets containing more than one cell, a light chain-specific PCR amplicon was obtained for 241, with 77 specific ones for mAb108 and 179 specific ones for OKT3 (15 droplets contained co-encapsulated mAb108 and OKT3, data not shown). This indicates an enrichment from 0.3 to 32%. In addition, the approximate output of ~1,000 mAb108-containing droplets within the dispensing gate of the 1:100 mixture correlated well with extrapolated ~75,000 theoretical hits in a preceding 1:1 mixing experiment (Figure 3c vs Figure 3bFigure 3; calculations not shown). Taken together, we showed that a setup using prelabeled ASCs for sorting toward cellular binding can be applied successfully.

Of note, the combination of a secretion FRET detection via a red channel (as before, Figure S2) in combination with green maximum fluorescence cellular binding sorting resulted in 80%

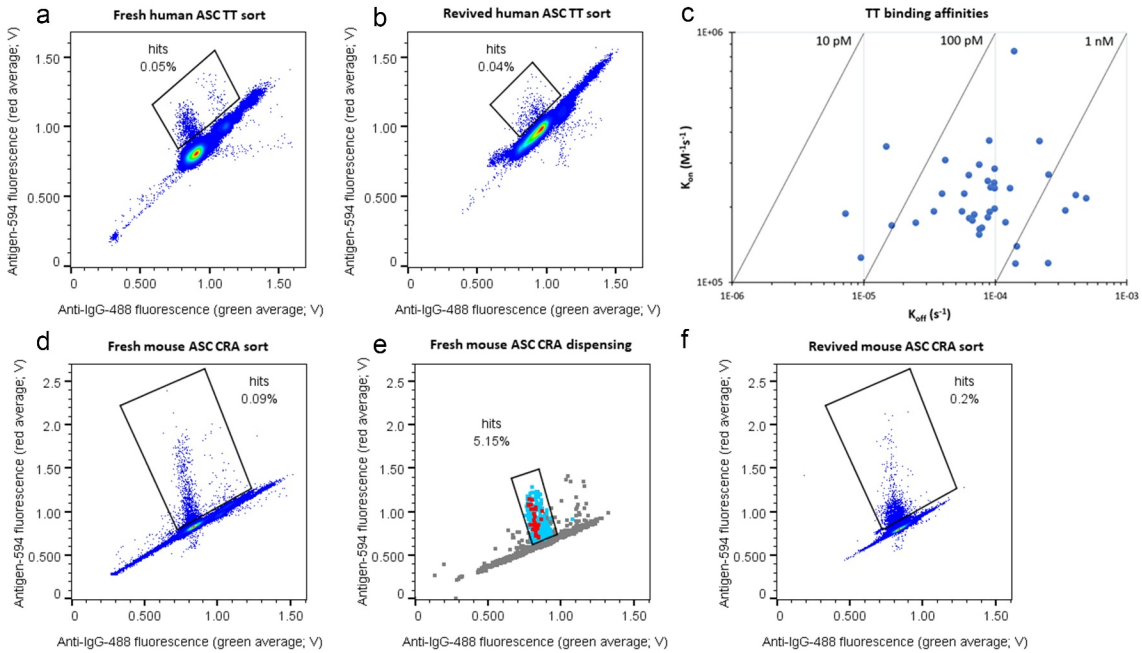

Figure 2. Sorting human and murine ASCs for target specificity. (a) Sort of freshly obtained human plasma blasts for secreted antibodies specific to tetanus toxoid (TT-CTD) via FRET. Decreased green donor and similarly increased red acceptor fluorescence indicate FRET by specific recombinant target binding in the respective droplet. (b) Sort for TT specificity as in (a), applying frozen and revived plasma blasts. (c) Confirmation of mainly sub-nanomolar TT affinities obtained via BLI within 4 weeks from ASC sourcing. (d) Sort of freshly obtained murine plasma cells for secreted antibodies specific for a cancer-related antigen (CRA). (e) Dispensing of sort shown in (d) – binders later confirmed as positive are shown in red, dispensed droplets in light blue, and non-dispensed droplets in gray that were used as a negative reference signal during dispensing. Positions of positive signals confirm appropriate gating. (f) Sort for CRA specificity as in (d), applying frozen and revived plasma cells. Refer to Table 1 for statistics of these antibody hit discovery campaigns.

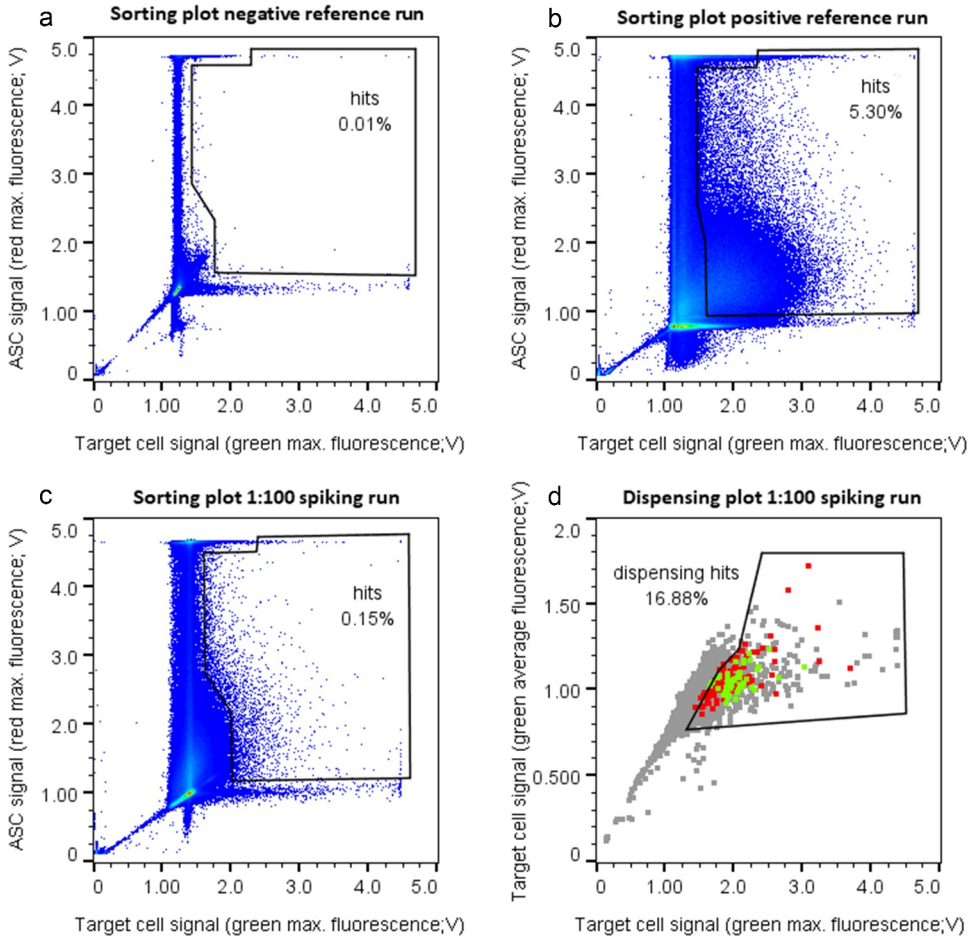

Figure 3. Proof-of-concept cellular screening data. Anti-EGFR specific mAb108 hybridomas were spiked into irrelevant OKT3 hybridoma, co-encapsulated with EGFR-positive A431 cells, and enriched by screening for cellular binding via the green channel. Hybridomas were anti-CD138 labeled for detection in the red channel to reduce false-positive sorting of droplets containing target cells only. Sorting gates were set to include red max. and green max. signals above background defined by a negative run, followed by dispensing in green max. vs. average mode. (a) Sorting plot of negative run including all reagents but OKT3 hybridoma only. (b) Sorting of positive reference run applying mAb108 and OKT3 in 1:1 ratio. (c) Sorting plot of 1:100 spiking run. (d) Dispensing of sort shown in C gating in green max. vs. average mode. Of 241 cell-containing droplets analyzed by PCR, 77 contained mAb108, equaling 32% and indicating successful enrichment. Signal distribution of mAb108 samples (green) vs. OKT3 (red) throughout the entire dispensing (and sorting, not shown) gate indicates appropriate gate definition.

loss of ASC detection, possibly due to competition of both detection methods, and hence, this approach could not be used (Figure S5).

Function-first antibody screening

In addition to the identification of antibodies with distinct binding specificities such as disease-related neoepitopes, many envisioned modes of action include a function that could be screened for high throughput microfluidics approaches when compatible reporter assays are available. We chose a CD3 agonist screening setup due to the long-standing yet high interest for next-generation antibody engineering, as well as ASC and reporter cells availability. For this, OKT3 secreting hybridoma were spiked in various dilutions into irrelevant mAb108 hybridoma secreting an EGFR-specific antibody, and enrichment after screening for the agonistic function reported by T-cell receptor signaling-induced green fluorescent protein (GFP) fluorescence was assessed. Calculation of the OKT3 dilution factor included the portion of hybridoma that actually secreted OKT3 antibody (55%) as determined by ELISpot (data not shown), e.g., an initial 1:100 OKT3 in mAb108 dilution resulted in 1:180 OKT3 secreting cells and hence 0.5556%. Identification of optimal in-droplet assay conditions represented a challenge. Optimization in bulk culture prior to microfluidic runs included the following five aspects to consider (Figure S6): (1) an ASC-to-reporter cell ratio of 1–6 million per milliliter input ensured co-encapsulation of one ASC, but at least one reporter cell per droplet, without applying too many reporter cells that could use up nutrients, leading to reduced viabilities and potentially false-positive signals; (2) optimal assay medium as well as choice and concentration of a recombinant anti-CD28 co-stimulatory antibody added to the solution enabled incubation for up to 12 hours; (3) 8 hour incubation time allowed a high signal-to-noise ratio while retaining cell viability; (4) ASC detection by prestaining enabled exclusion of ASC-free high reporter cell background events during sorting; and (5) maximum fluorescence (peak mode) sorting, as comprehensively introduced recently.[20] Adjusted dispensing gains for reporter readout with a broad dynamic range allowed appropriate gating (Figure 4c) of droplets containing agonistic antibody secreting cells.

The number of ~2000 – 4000 dispensed droplets in spiking experiments was in the expected range (accounting for the respective spiking dilution, ASC/reporter cell co-encapsulation, loss during droplet generation or flow stabilization for sort, and dispensing; Figure S7) and indicated broad coverage of the applied diversity. After factual dilution to 0.6% and sorting for the agonistic function measured by induced reporter cell GFP fluorescence, 192 of 1936 droplets in the dispensing gate were analyzed by PCR for the presence of hybridoma [applying an unselective heavy chain variable domain (VH) primer] and mAb108 or OKT3 [via specific kappa light chain variable domain (VK) primers]. Of 192 samples, 147 VH amplicons (PCR recovery rate 77%) and 130 OKT3-specific VK amplicons were observed, equaling a hit rate of 68% in all dispensed samples and an enrichment to 88% of all resulting antibody hits (excerpt shown in Figure 4d).

Throughout all campaigns reported here, the process times from cell preparation to full antibody gene recovery and to confirmed recombinant antibodies were two days and four weeks, respectively. The workflow can be split after PCR antibody gene recovery, allowing flexibility upon resource restrains or a staggered approach of paralleled screening, recombinant reproduction, and confirmatory analyses when distinct resources are available.

Discussion

Microfluidics offer advantages, such as speed, native chain pairing, direct screening for selectivity, cellular binding, or desired function, and have recently been used for diverse activities directed toward therapeutic antibody discovery. While broad diversities of neutralizing antibodies against infectious diseases have been identified from convalescent individuals,[22,29,30] microfluidics can also yield antibodies for cancer or immunological diseases.[31,32] Although the identification of screening tools[16] or production cell lines[20] was not within the scope of this study, successful screening for high secretion could be shown for human plasma blasts, murine plasma cells, and hybridoma (Figure S2), similar to published work.[20]

Several proof-of-concept studies for antibody screening toward high specificity to a recombinant antigen have used TT, pioneered for human diversities by Lanzavecchia and coworkers in combination with low throughput plate-based

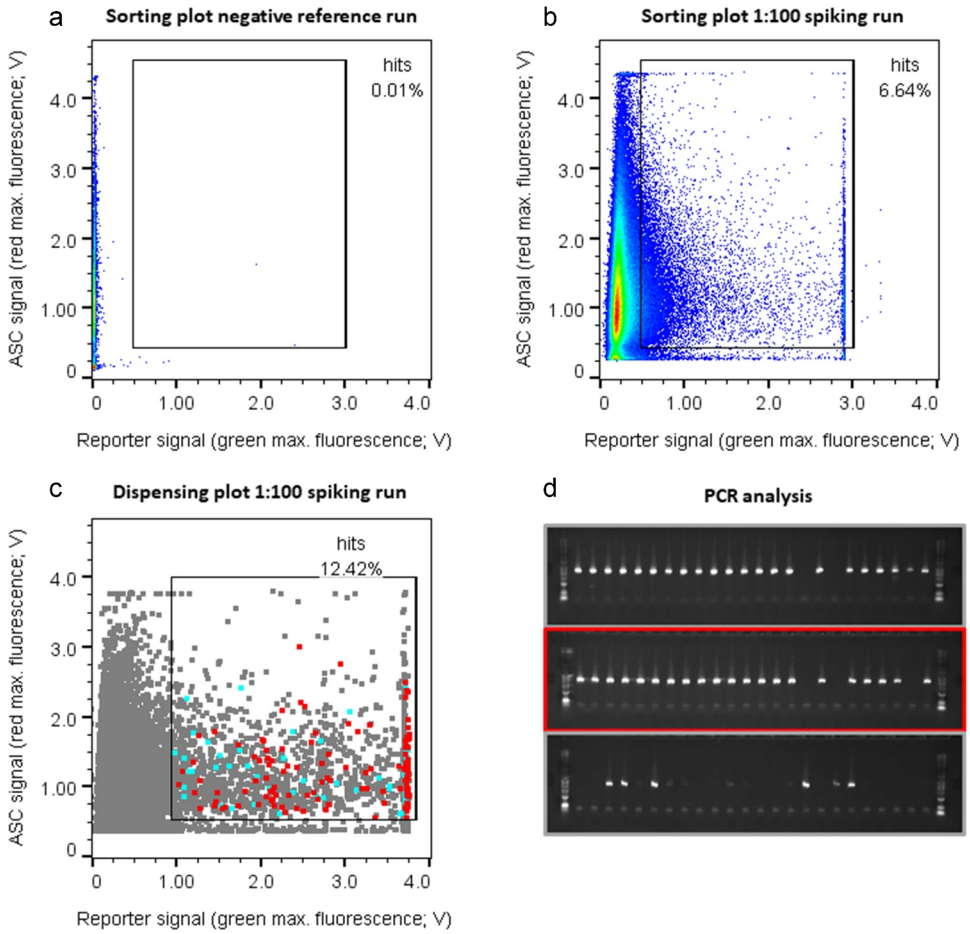

Figure 4. Functional screening proof of concept. Agonistic OKT3 hybridomas were spiked into irrelevant mAb108 hybridomas, co-encapsulated with Jurkat-GFP reporter cells, and enriched by screening for CD3 agonistic function induced GFP fluorescence in green max. fluorescence sorting. (a) ASC and reporter signal at 0 h. (b) Sorting reporter signal after 8 h incubation. A broader gating strategy included 6.6% events, enabling more stringent gating during dispensing. (c) Dispensing plot. Gating 12% events (of 6.6% sorted) equals approximately 0.55% expected positive events. Droplets not evaluated in gray, dispensed cells in light blue, and confirmed OKT3 containing droplets in red. Observation of positive events within the full gate area indicates theappropriate gating setup. (d) Exemplary single well PCR recovery gel excerpt indicating the presence of antibody genes (VH primers detecting both OKT3 and mAb108) in the top lane, OKT3 VK specific amplicons in the middle lane, and mAb108 VK specific amplicons in the lower lane, indicating an enrichment of OKT3 clones.

screens,[34] as well as recently for interrogation of mouse immune diversities combined with high throughput microfluidic screens.[20] Rapid identification of a broad 44 TT-specific human antibody panel (Figure 2, Table 1) exemplifies the capability of the workflow we developed for direct identification of both high affinity and specificity antibodies from the human peripheral blood. A similar setup for the interrogation of human disease diversities, such as from cancer or autoimmune patients,[35] could yield highly valuable therapeutic hit candidates in a short time. While this seems desirable for future development, current antibody hit discovery is often based on immunization of transgenic or wild-type rodents. Here, immunization of wild-type mice with CRA in combination with plasma cell preparation and droplet microfluidics allowed identification of 30 sequence-diverse CRA-specific antibody hits. A general advantage of microfluidics compared to hybridoma or display technologies is speed while retaining similar or enabling higher throughput.[36] Specifically, in the cases presented herein, the process time from cell preparation to confirmed recombinant antibody panels was four weeks, in line with the timelines of similar microfluidic approaches.[16]

ASC screening toward specific cellular binding using microfluidic approaches can facilitate identification of hits against complex targets, e.g., membrane spanning receptors, as use of recombinant target protein or generation of membrane-like particles such as baculovirus particles can be avoided in the screening process. Similar to the setup demonstrated and in detail described recently,[20,37] a cellular binding screen enriched spiked anti-EGFR hybridoma for binding to EGFR-positive A431 tumor cells from 0.3 to 32% indicated sorting capacity and success of additional ASC prelabeling to reduce false-positive events.

Direct antibody screening for the desired function (addressing known targets or agnostic of those in pure phenotypic screens[7,38–40]) in a high throughput manner could increase the likelihood for best-in-class antibody discovery. Pioneering early works mainly using autocrine, i.e., self-labeling, one cell systems[41–44] or mammalian display, with focus on antibody developability such as affinity maturation[45] and beyond,[5] are all compatible with flow cytometry for high throughput variant screening. The advent of microfluidics broadened opportunities for functional screening of secreted antibodies for internalization and intracellular signaling,[20] virus neutralization,[29] optimal agonistic properties,[28] and further biotherapeutics[23] when combined with suitable, mostly fluorescence-based cellular reporter assays. In evaluating function-first screening capacities, the anti-CD3 agonist antibody OKT3-secreting hybridoma cell line was spiked into irrelevant antibody-secreting hybridoma and could be highly enriched from 0.55% to 88% in a single run sorting for the GFP reporter signal (Figure 4), indicating successful screening toward the agonistic function. This represents, to our knowledge, the first example of a broad functional full IgG antibody screening in droplets.

Although the hit discovery processes described here and recently elsewhere[16,20] rapidly deliver broad antibody panels, several levers for improvement remain to be optimized. In the case of immune library screening, optimization of initial immunization steps as a basis for successful ASC sourcing may include an adapted protocol[32] for the respective host species, the sampling time, and interrogated organs.[10,46] Although fresh ASC sampling may be beneficial for the highest secretion and resulting hit rates,[31] methodologies for cryopreservation retaining the functionality of lymphocytes[47] may allow for the flexibility to decouple cell preparation and ASC screening. ASC diversities optimized in these ways could be used in a multitude of recently matured microfluidic approaches representing differing underlying screening technologies. While several companies offer microfluidic chips of flexible design and antibody screening campaigns,[12,20,29] standalone devices applicable to academic and commercial antibody hit discovery are also available.[16,33,48] A comprehensive review by Fitzgerald and Leonard covers the multitude of current ASC screening approaches for antibody discovery.[13]

Several properties of the Sphere Fluidics (Cambridge, United Kingdom) "Cyto-Mine" device[33] affect the overall output. An exchangeable cartridge design allows for ease of handling and avoids cross-sort contaminations. The droplet-based setup with constrained throughput of two million droplets allows screening of approximately 720,000 cells for recombinant protein binding, and ~256,000 for cell binding is lower than those reported for the "DropMap"[20,31] and "PRESCIENT"[29] approaches or in the work by the Merten group,[12] but likely sufficient for full coverage of single donor immune diversities. Gel microdroplet-fluorescence-activated cell sorting approaches could allow higher throughput screening of larger recombinant secretion libraries,[23,37] as well as reagent washing or extraction, and hence multi-step assays. Droplet-based approaches are generically limited to closed homogeneous fluorescence-based readouts. While various assays can be applied, the mandatory choice for one readout per run excludes multi-paralleled evaluation of cross-reactivities or off-target binding broadly applied during, for example, primary ELISA-based phage display screens. Cellular readouts such as cellular binding or manipulation of cellular functions require Poisson-distributed co-encapsulation of ASCs with target cells. Co-encapsulation of mainly single ASCs with at least one target cell could be achieved by optimized cell ratios, and the immanently reduced number of ~256,000 ASCs to be screened remained sufficiently high to support full diversity screening campaigns.

The current device setup comprises 488 nm excitation, allowing simultaneous green and red channel readouts by the application of tandem dyes or FRET pairs, yet remains limited compared to approaches offering more fluorescence channels for higher multiplexing capabilities.[49] In concert with flexible flow cell design,[20,29,31] customizable solutions offer broader assay compatibility. In contrast, ASC compartmentalization in nanostructures such as nanopens[16] or microcapillaries[17–19] offers the advantage of recording multiple readouts for the same ASC, for example, consecutively interrogating for secretion, recombinant and cellular binding, or function. As the throughput of these approaches is often rather tens of thousands than one million, the choice of the best-suited technology will in most cases be driven by the intended throughput versus multiplexed readout options. The Cyto-Mine screening process allows for dispensing in either bulk or single well export, combined here with a rapid post-dispensing

downstream single cell cloning workflow, similar to B cell cloning, for fast confirmation in in-format secondary screens. In contrast, re-compartmentalization for NGS analyses of IgG repertoire diversity, clonal expansion, and somatic hypermutation for educated re-synthesis and production of fewer antibodies[20] is a more laborious but viable option that may open up avenues for enhanced antibody engineering, such as improving target specificity by predictive models of antibody binding learned from high-throughput experimental data.[50]

Microfluidic approaches facilitate the analysis of the spatial and temporal progression of the immune response in different species,[8,10] allowing deeper insights that can be applied to enhanced identification of potent therapeutic antibodies. In case studies presented here, IgG repertoires derived from large numbers of rodent plasma cells or human plasma blasts were screened at high throughput using a wide range of assays, including cellular and functional readouts. The broad datasets obtained with a stand-alone device exemplify the versatility of microfluidics for rapid antibody discovery. Future development warrants optimization of several device properties, workflow steps, and methodologies, as discussed above. Application of homogeneous monoclonal secretion libraries[28] could enable repeated interrogation of the full B cell diversity in robust in-format screens. Stand-alone devices[16,33] may accelerate the full establishment of microfluidics as a standard methodology for the discovery of differentiated therapeutic antibody candidates with unique properties, identified in broad functional or target-agnostic phenotypic screens[7] against diverse antigens, including those that are difficult to target.

Materials and methods

Recombinant proteins, detection antibodies and their labeling

Recombinant tetanus toxoid (C-terminal domain, termed TT-CTD herein) used in antibody selection was kindly provided by preclinics GmbH, Potsdam, Germany, and fluorescently labeled with Alexa Fluor (AF)™ 488 Antibody Labeling Kit (ThermoFisher Scientific, Waltham, Massachusetts, USA), according to manufacturer's protocol. Tetanus toxoid for biolayer interferometry (BLI) affinity determination was sourced from Enzo Life Sciences GmbH, Lörrach, Germany, and Calbiochem (Merck KGaA, Darmstadt, Germany). Biotinylated recombinant extracellular domain of a CRA was produced at Merck Healthcare KGaA, Darmstadt, Germany.

Target, reporter, and antibody-secreting cells

OKT3 (anti-human CD3, catalog number (cat.) 86022706–1VL, Sigma-Aldrich, St- Louis, Missouri, USA) antibody-expressing mouse hybridoma cells were cultured in Iscove's Modified Dulbecco's medium (IMDM with L-glutamine; Gibco by Life Technologies, Carlsbad, California, USA) with 20% fetal bovine serum (FBS). mAb108 (anti-human EGFR, cat. HB-9764, ATCC, Manassas, Virginia, USA) antibody-expressing mouse hybridoma and EGFR-expressing A431 epidermoid carcinoma (ATCC) cells were cultivated in Dulbecco's modified Eagle's medium (DMEM high glucose, Sigma-Aldrich) with 10% FBS. For functional screening or cellular binding assays, hybridoma cells were blocked with purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block™) Clone 2.4G2 (RUO) from BD Biosciences, Franklin Lakes, New Jersey, USA. The Jurkat-GFP reporter cell line (NF-κB/Jurkat/GFP™ Transcriptional Reporter Cell Line) was sourced from SBI System Bioscience, Palo Alto, California, USA, and cultured in RPMI 1640 medium (Sigma-Aldrich) with 10% FBS heat inactivated and 1x Glutamax. Primary mouse plasma cells (bone marrow, lymph nodes, splenocytes) were prepared five-days post boost with TT-CTD or CRA and shipped overnight on blue ice from preclinics GmbH. Cells were enriched for ASCs by EasySep™ Mouse CD138 Positive Selection Kit (StemCell, Vancouver, Canada) according to the RoboSep manual.

Human plasma blasts were isolated from 297 mL peripheral blood (with ethylenediaminetetraacetic acid) of healthy donors (medical officer department, Merck KGaA, in accordance with Merck internal guidelines and the Declaration of Helsinki), donated 6- or 7-days post TT vaccination. Preparation of peripheral blood mononuclear cells (PBMCs) was performed following the SepMate™-50 (IVD; StemCell) manual using 15 mL of Lymphoprep (StemCell) as the density gradient medium. Briefly, 15 mL of the washing buffer (1x Dulbecco's phosphate buffered saline + 10% FBS heat inactivated, both from Sigma-Aldrich) was added into SepMate tubes, followed by addition of 20 mL peripheral blood. Samples were centrifuged at 1200x g for 10 min with brake off. Before transfer of the layer with enriched PBMCs, detached cells were removed from the tube wall with a cell scraper. After a spin down (300x g, 8 min, brake on) of the enriched PBMCs, cells were washed twice with one platelet-removing centrifugation step. Washed and isolated PBMCs were used for Pan-B enrichment with EasySep™ human Pan-B cell enrichment kit (StemCell) via RoboSep (StemCell). Then, CD38 positive cells were separated using a CD38 monoclonal antibody (HIT2), Biotin, eBioscience™ (Invitrogen, Waltham, Massachusetts, USA), in a concentration of 0.5 µg/100 µl in combination with EasySep™ human biotin positive selection kit II (StemCell). Finally, human CD38+ or murine CD138+ cells were transferred to IMDM, GlutaMAX™ supplement medium (Gibco by Life Technologies) supplemented with 10% FBS heat inactivated (Sigma-Aldrich), 1x non-essential amino acid solution (NEAA; Gibco by Life Technologies), 1x penicillin-streptomycin (Pen/Strep; Sigma-Aldrich), 1x sodium pyruvate (Gibco by Life Technologies), and 1x 2-Mercaptoethanol (Gibco by Life Technologies).

Storage and revival of B cells

Cells not directly applied to microfluidic applications were stored in liquid nitrogen. For this, remaining cells were spun down at 500 x g for 5 min, the supernatant was discarded, and cells were resuspended in CryoStor CS10 (StemCell). Cryogenic vials (ThermoFisher Scientific) containing ~ 2×10^6 viable cells/mL were placed in a Nalgene Cryo 1°C freezing container at −80°C overnight and afterward transferred to liquid nitrogen for long term storage. For thawing, cryo-vial was warmed in a 37°C water bath for ~5 min. Cells

were then transferred in new 50 mL centrifugation tube and slowly filled with pre-warmed cell medium as recently described by Fecher *et al.*[47] After a 5 min, 500 x g spin down, cell pellet was resuspended in fresh cell medium.

ELISpot

MultiScreen$_{HTS}$ IP Filter Plates, 0.45 µm (Sigma-Aldrich) were precoated with 25 µl per well of 70% ethanol, incubated for 1 min at room temperature, washed twice with 50 µL 1x phosphate-buffered saline (PBS; Sigma-Aldrich), and supernatant was discarded. Plates were coated with 100 µl of goat anti-mouse IgG H + L antibody (20–30 µg/mL; cat. 115–005-003, Jackson ImmunoResearch, West Grove, Pennsylvania, USA) overnight at +4°C, then washed twice with 200 µl 1x PBS and blocked with 200 µl of cell cultivation medium (containing FBS) for 2 h at 37°C in an incubator with 5% CO_2. 200 µl of serial cell dilutions were transferred into the plate and incubated overnight without shaking at 37°C, 5% CO_2 and 80% humidity. Subsequently, cells were removed, and plates washed three times with 150 µl wash buffer (1xPBS + 0.05% Tween20). 100 µl detection antibody goat anti-mouse F(ab')$_2$ specific-peroxidase (cat. 115–035-072, Jackson ImmunoResearch) at 1 µg/mL in PBS + 5% FBS was added and incubated overnight at +4°C. For developing, the plates were washed three times with 150 µl wash buffer, while AEC staining solution (Sigma-Aldrich) was prepared in Milli-Q-water (12 mL Milli-Q-water, 6 drops Vial 1 acetate buffer, 3 drops Vial 2 AEC chromogen and 3 drops Vials 3 3% hydrogen peroxidase, sterile filtrated), and 75 µl were transferred to each well for 30 min incubation. Plates were washed twice with 200 µL PBS and once with 200 µl Milli-Q-water, dried, and read on an ELISpot-reader (AID, Straßberg, Germany).

Microfluidics-assisted ASC sorting

For each Cyto-Mine run, the encapsulation medium was prepared freshly by combining the 830 µL cell cultivation medium with 160 µL of OptiPrepTM density gradient medium (Sigma-Aldrich) and 10 µL of 10% PluronicTM F-68 (Gibco by Life Technologies). Depending on the assay, different amounts of detection antibodies, labeled antigens, and other necessary reagents were applied. In FRET-based experiments (screening for antibody secretion and recombinant antigen binding), DyLight-488/AlexaFluor488 labeled antibodies/antigens were used as the donor and DyLight-594/AlexaFluor594 labeled reagents as the acceptor in adapted concentration ratios. These reagents were added to reach 1 mL of the basic encapsulation medium used for resuspension of cells of interest. 1 million ASCs were used for secretion and recombinant antigen assays and for cell surface binding or functional screenings, and 4 or 6 million target cells were additionally added to ensure a high percentage of target cell presence in each droplet. Before loading the Cyto-Cartridge® (Sphere Fluidics), cell suspension was filtered through 40 µM Flowmi® cell strainer (Bel-Art SP Scienceware, Wayne, New Jersey, USA). Cells in 1 mL were encapsulated in 2 million droplets and incubated at 37°C for 2–18 h, depending on the respective assay setup. Sorting and dispensing in 96-well plates were conducted following the manufacturer's protocols. In sorting, it was necessary to sort at least 15,000 droplets for good system performance, so in some cases, positive droplets were filled up with negative ones. All sorted droplets were stored in a collection chamber and then analyzed again and dispensed in dispensing mode. Due to different photomultiplier tube settings, plots did not conform in sorting and dispensing mode. Gates for both sorting and dispensing were defined to include ≤0.01% events in preceding negative runs (see, for example, Figure 3a and Figure 4a) by excluding populations of empty single, doublet, or triplet droplets.

For the analysis of human IgG secreting cells, Goat Anti-Human IgG Fc-DyLight® 488 (cat. ab97003, Abcam, Cambridge, United Kingdom) as the donor and Goat F(ab')2 Anti-Human IgG – (Fab')2 (DyLight® 594), pre-adsorbed (cat. ab98602, Abcam), as the acceptor were used as detection antibodies in a ratio of 2 µg/mL (donor) and 40 µg/mL (acceptor). Human antibodies against TT-CTD were screened with AF488 labeled TT-CTD (preclinics) and Goat F(ab')2 Anti-Human IgG–(Fab')2 (DyLight® 594), pre-adsorbed (cat. ab98602, Abcam) in a ratio of 5–20 µg/mL. Mouse anti-CRA binders were detected via biotinylated CRA (2.8 µg/mL), Streptavidin-AF488 (20 µg/mL, cat. S32354, Life Technologies, Carlsbad, California, USA), and Goat F(ab')2 Anti-Mouse IgG – (Fab')2 (DyLight® 594), pre-adsorbed (40 µg/mL, cat. ab98759, Abcam).

For the selection of cell surface binding antibody secreting cells, 5 µg/mL PE/Dazzle 594 rat anti-mouse CD138 antibody (cat. 142528, BioLegend, San Diego, California, USA) was applied for ASC labeling along with 2.5 µg/mL AF488 AffiniPure Fab Fragment goat anti-mouse IgG (H + L) (cat. 115–547-003, Jackson ImmunoResearch) for target-specific monoclonal antibody (mAb) detection on the cell surface and 1 µg/mL Mouse BD Fc Block (cat. 553142, BD Biosciences). Hybridoma were Fc blocked following the manufacturer's protocol, premixed (e.g., 1:1 or 1:100), and combined with six-fold excess of target cells. Maximum fluorescence signals were recorded applying the "peak mode" setting.

For functional screenings, OKT3 and mAb108 hybridoma cells were stained with 5 µM CellTracker™ Orange CMRA Dye (cat. C34551, ThermoFisher Scientific). No further detection antibodies were required. Stained ASC and Jurkat-GFP cells were co-encapsulated with 5 µg/mL costimulatory anti-human CD28 antibody (clone 28.2, cat. 302902, BioLegend).

Single cell mAb gene recovery and subcloning

Positive droplets from Cyto-Mine were dispensed in 10 µL lysis buffer [1 µL of 0.1 M dithiothreitol (DTT, Invitrogen), 0.5 µL of RNaseOUT™ recombinant ribonuclease inhibitor (Invitrogen), 8.5 µL of nuclease-free water (Invitrogen)] in a 96-well PCR plate. Dispensed plates were stored at −80°C. Six microliters of denaturing master mix was added to each well and incubated for 5 min at 65°C in a thermal cycler. Denaturing master mix contained 0.58 µL of 50 µM random hexamers (Invitrogen), 0.34 µL of RNaseOUT™ recombinant ribonuclease inhibitor, 1 µL of 10% IGEPAL CA-630 (Sigma-Aldrich), and 4.08 µL of nuclease-free water. Then, 14 µL of master reverse transcription (RT) buffer was transferred to the PCR plate, containing 5.58 µL of 5x SuperScriptIV buffer, 2 µL of 0.1 M DTT, 0.66 µL

of SuperScript™ IV reverse transcriptase (Invitrogen), 0.84 µL of 10 mM dNTP mix (Life Technologies), 0.42 µL of RNaseOUT™ recombinant ribonuclease inhibitor, and 4.5 µL of nuclease-free water. After a short spin down (1500 rpm, 30 sec), RT was performed in a thermal cycler (Bio-Rad, Hercules, California, USA or Bioer Technology, Hangzhou, Zhejiang, China) under the following conditions: 20°C for 15 min, 42°C for 5 min, 25°C for 10 min, 42°C for 10 min, 90°C for 10 min, and hold on 4°C. cDNA was used directly or stored at −20°C.

Twenty five microliters of Platinum™ II Hot-Start PCR Master Mix (2X, Invitrogen) was mixed with 0.5 µL of forward primer and 0.5 µL of reverse primer (Table S1) with a stock concentration of 10 µM and filled with nuclease-free water to a total of 45 µL per well. For VH, VK, and VL (lambda light chain variable domain), separated master mixes were created with individual primers. Five of each cDNA were added to each prepared well of a 96-well PCR plate to perform the first PCR. PCR amplification was performed with the following settings: initial denaturation 95°C for 2 min, 30 cycles of 95°C for 30 sec denature, 55°C (VH) or 50°C (for VK/VL) for 30 sec anneal, 72°C for 40 sec extend, and a final elongation at 72°C for 10 min.

Five microliters of PCR amplicon of the first PCR was used for nesting PCR (2nd PCR) with still separated V-Genes. The nesting PCR master mix contained 5 µL of 10x AccuPrime PCR buffer 1, 0.2 µL of AccuPrime™ Taq DNA polymerase, high fidelity (Invitrogen), 0.1 µL of forward primer and 0.1 µL of reverse primer (100 µM stock; Table S1), and 39.6 µL of nuclease-free water for each well. PCR reactions were performed in Thermal Cyclers with the subsequent program settings: 1 cycle at 95°C for 3 min and 5 cycles with 94°C for 30 sec, 42°C for 30 sec, and 72°C for 30 sec, followed by 50 cycles with 94°C for 30 sec, 55°C for 30 sec (for VH, VK, and VL), and 72°C for 40 sec and finalized by 72°C for 5 min.

PCR products were detected on E-Gel™ 48 agarose gels, 2% (Invitrogen), by loading 5 µL of the product onto each line and a runtime of 15 min. Perfect DNA™ Markers, 0.05–10 kbp – Novagen (Merck KGaA), was used as the reference.

All hits were re-arrayed into a fresh PCR plate and purified using a Wizard® SV 96 PCR Clean-Up System (Promega, Madison, Wisconsin, USA) with 95% of ethanol for wash steps (PCR amplicon <500 bp), according to the kit manual. Samples were eluted with 50 µL of nuclease-free water. All PCR amplicons were reformatted into a human pTT-IgH, pTT-IgK, or pTT-IgL vector backbone, respectively. One microliter of of either vector was pipetted into 96-well PCR plate wells on ice and 1.5 µL of the purified VH, VK, or VL amplicons were added, respectively. The reactions were incubated for 10 min on ice. Meanwhile, MultiShot™ StripWell Mach1™ T1 Phage-Resistant Chemically Competent *E. coli* (Invitrogen) were thawed on ice. Twenty five microliters of competent cells were added to amplicon-vector recombinants. After an incubation of 30 min on ice, plates were heat-shocked at 42°C for 45 sec in a preheated water bath and again on ice for 3 min. Room-temperature S.O.C. medium (175 µL) was added to plates and the whole mix was transferred to a new 96-deep well plate (KingFisher™ Plastics for 96 deep-well format, ThermoScientific). With AeraSeal film (Sigma-Aldrich), covered plates were incubated for 1 h at 37°C, 70% humidity, and 750 rpm. After incubation, 15 µL of this pre-culture were transferred to 1 mL of Super Broth medium (Teknova, Hollister, California, USA) with 100 mg/L of ampicillin and incubated again for 18 h with the same conditions. Plasmid purification was done with QIAprep 96 Turbo Kits (Qiagen, Hilden, Germany) according to the manual.

Small-scale production in Expi293F

Two days prior to transfection with ExpiFectamine™ 293 Transfection Kit (Gibco by Life Technologies), Expi293F cells (Life Technologies) were seeded with a cell titer of 1.5×10^6 viable cells/mL in an adequate flask and incubated at 37°C, 80 rpm, 80% humidity, and 5% CO_2. On the day of transfection, cells were counted, and the required amount was centrifuged and suspended in a fresh Expi293 Expression Medium (Gibco by Life Technologies) with a cell titer of 2.9×10^6 viable cells/mL. Cell suspension (540 µL) was transferred to each well of a KingFisher™ Plastics for 96 deep-well-plate, covered by an AeraSeal film, and further incubated until needed. Meanwhile, 176 µL of ExpiFectamine was pre-mixed with Opti-MEM™ I Reduced Serum Medium (Gibco by Life Technologies) and incubated for 5 min. The volumes for the 600 ng heavy chain and 600 ng corresponding light chain plasmids were filled to 30 µL with Opti-MEM medium. Diluted DNA was mixed by pipetting with 30 µL of diluted ExpiFectamine and incubated for 20 min at room temperature. DNA complexes were added to the prepared cells (60 µL of DNA complex containing one heavy chain and the corresponding light chain plasmids + 540 µL of cell suspension) and mixed gently. Sealed plates were incubated on a shaker platform at 1000 rpm, 37 °C, 80% humidity, and 8% CO_2. After 24 h, 3 µL enhancer 1 and 30 µL enhancer 2 were added per well. Five to seven days after transfection, the supernatant was collected and used for further analysis.

Biolayer interferometry

Kinetic measurements were performed on an Octet RED96 system (ForteBio, Pall Life Science, New York, USA). For analysis of anti-TT antibodies, loading on anti-human Fc biosensors was conducted for 5 min with (1) supernatant from transfected Expi293F undiluted, (2) the TT-reference antibody (Human Anti-Tetanus Toxoid Recombinant Antibody, clone B7-15A2, creative biolabs), or (3) an irrelevant antibody with a concentration of 4 µg/mL. A 30 sec rinse in kinetics buffer (PBS + 0.1% Tween-20 + 1% BSA) for baseline definition was followed by association to 50 nM TT for 300 sec and dissociation for 600 sec in kinetics buffer.

For evaluation of anti-CRA specificity, biotinylated CRA (3 µg/mL) was loaded on streptavidin biosensors for 200 sec followed by 30 sec in Expi293 Expression medium + 1% FBS (Gibco by Life Technologies) as a baseline step. Then, association in the undiluted supernatant was measured for 300 sec and dissociation in the cell medium containing FBS for 600 sec. Anti-CRA and an irrelevant antibody (both 50 nM and from

Merck Healthcare KGaA) were used as positive control and negative reference, respectively. All steps were performed at 25°C and 1000 rpm. Data were analyzed with ForteBio data analysis software 8.2 and 12.0 using a 1:1 binding model after Savitzy-Golay filtering.

Sequence confirmation and analyses

Sanger sequencing was used for sequence analyses, and the resulting forward + reverse consensus sequences were analyzed with Geneious Prime software version 2020.0.5.

Abbreviations

- AF, Alexa Fluor
- ASC, antibody secreting cell
- BLI, biolayer interferometry
- CRA, cancer related antigen
- DMEM, Dulbecco's Modified Eagle's Medium
- EGFR, epidermal growth factor receptor
- ELISpot, enzyme-linked-immuno-spot
- Fab, antigen-binding fragment
- FBS, fetal bovine serum
- Fc, crystallizable fragment
- FRET, Förster resonance energy transfer
- GFP, green fluorescent protein
- H+L, heavy and light chain
- HTS, high throughput screening
- IMDM, Iscove's Modified Dulbecco's Medium
- mAb, monoclonal antibody
- MACS, magnetic-activated cell sorting
- NEAA, non-essential amino acid solution
- NGS, next generation sequencing
- PBMC, peripheral blood mononuclear cells
- PCR, polymerase chain reaction
- Pen/Strep, penicillin-streptomycin
- RT, reverse transcriptase
- TT, tetanus toxoid
- TT-CTD, Tetanus toxoid C-terminal domain
- VH, heavy light chain variable domain
- VK, kappa light chain variable domain
- VL, lambda light chain variable domain

Acknowledgments

The thank Qi An, Shang-Pu Tsai, Qingyong Ji, and Desislava Yanakieva for methodology input and support; Laura Unmuth for CRA BLI; Serena Davoli, Wilna Gray, Darren Matthews, Peter Kennedy, and colleagues at Sphere Fluidics for Cyto-Mine support; Philipp Haselmayer and Shinji Okitsu for advice on human plasma blast preparation; Daniela Noack and Christine Drechsler for IncuCyte support; Rebecca Harth for ELISpot; and Melanie Seck and Julia Hanschke for RoboSep and blood purification. Figure 1 was created using BioRender.com.

Disclosure statement

The authors declare no conflict of interest.

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