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Rapid single B cell antibody discovery using nanopens and structured light

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ABSTRACT

Accelerated development of monoclonal antibody (mAb) tool reagents is an essential requirement for the successful advancement of therapeutic antibodies in today's fast-paced and competitive drug development marketplace. Here, we describe a direct, flexible, and rapid nanofluidic optoelectronic single B lymphocyte antibody screening technique (NanOBlast) applied to the generation of anti-idiotypic reagent antibodies. Selectively enriched, antigen-experienced murine antibody secreting cells (ASCs) were harvested from spleen and lymph nodes. Subsequently, secreted mAbs from individually isolated, single ASCs were screened directly using a novel, integrated, high-content culture, and assay platform capable of manipulating living cells within microfluidic chip nanopens using structured light. Single-cell polymerase chain reaction–based molecular recovery on select anti-idiotypic ASCs followed by recombinant IgG expression and enzyme-linked immunosorbent assay (ELISA) characterization resulted in the recovery and identification of a diverse and high-affinity panel of anti-idiotypic reagent mAbs. Combinatorial ELISA screening identified both capture and detection mAbs, and enabled the development of a sensitive and highly specific ligand binding assay capable of quantifying free therapeutic IgG molecules directly from human patient serum, thereby facilitating important drug development decision-making. The ASC import, screening, and export discovery workflow on the chip was completed within 5 h, while the overall discovery workflow from immunization to recombinantly expressed IgG was completed in under 60 days.

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Introduction

The annual number of antibody-based therapeutics to receive approval in either the European Union or the United States reached double digits for the first time in 2017.[1] Moving such therapeutics from early discovery through clinical trials and eventually into the marketplace requires the development and validation of numerous analytical methodologies.[2,3] Ligand binding assays (LBAs) are analytical tools frequently used for pharmacokinetics (PK), pharmacodynamics (PD), and toxicokinetics studies due to their sensitivity, specificity, reproducibility, and relatively simple execution.[4–6] LBAs constructed with high-affinity, neutralizing, and paired capture/detection monoclonal antibody (mAb) reagents are particularly useful for correlating the levels of free-drug with efficacy of the therapeutic candidate.[3,6–8] Anti-idiotype LBAs offer significant advantages over generic anti-Fc or anti–light chain, polyclonal antibody–based assays owing to comparatively less nonrelevant human IgG constant region binding when directly testing human serum samples containing endogenous serum IgG.[8]

Tool antibodies for use in LBAs are most frequently derived from in vivo immunization followed by either immortalization as hybridomas[9,10] or as immune cell libraries for display technologies such as phage or yeast.[11] Despite these methodologies being important and reliable discovery engines, they have notable process limitations. Hybridomas require immortalization of antibody-secreting cells (ASCs) via somatic fusion to a myeloma cell line. Even with optimized electrofusion protocols, only 1 of 5000 input B cells survives fusion, becomes immortalized, and secretes antibody.[12] Additionally, hybridoma methods generally require extensive cell culture, which is labor intensive and dependent on mitosis, further slowing development timelines. Display technologies, which start from antigen-experienced IgG messenger RNA (mRNA) pools, suffer primarily from the loss of the cognate VH and VL pairing of the input repertoire.[11,13,14] This can result in the identification of low-affinity antibodies, and usually necessitates multiple rounds of time-consuming affinity maturation to generate potent molecules.[15] Finally, hybridoma and display technologies both require substantial laboratory space, aseptic and benchtop liquid handling automation, and multiple trained operators to perform antibody discovery campaigns at commercial scales.

Recently, several approaches that effectively used "micro tools" for direct B cell antibody discovery have been described.[16–18] Custom microfluidic chambers,[19] microencapsulation,[20,21] custom microwell devices,[22–24] and microcapillary tubes[25] have all been used to discover mAbs, although with variable success. These methods all allow the direct isolation and screening of ASCs as single cells, thereby

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allowing some phenotypic characterization of the encoded antibody without B cell immortalization or library generation. Recovery of the desired sequences is generally accomplished using single cell polymerase chain reaction (PCR)[26] or, in some cases, barcode-based next-generation sequencing followed by recombinant cloning and expression en masse.[27] These micro tools eliminate the need for immortalization, are species-agnostic, allow high throughput sampling as well as multiparameter phenotyping of the input cells, have reduced reagent consumption compared to hybridoma and display technologies, and maintain the ability to retain the native VH and VL pairings of the original antibody. Despite their many advantages, the aforementioned micro-tool techniques are not widely commercially available; lack the ability to execute back-to-back iterative screens; cannot keep the cells, and therefore the antibody mRNA, viable for extended periods of time; and require an operator to physically pick the individual B cells of interest in subsequent molecular recovery.

Here, we present a nanofluidic optoelectronic B lymphocyte antibody screening technique (NanOBlast) built around Beacon™, a commercially available (Berkeley Lights), integrated culture and imaging platform. Beacon™ leverages a novel, disposable, microfluidic culturing chip (OptoSelect™ OS3500) containing 3513 one nanoliter pens (hereinafter, nanopens) fabricated out of polydimethylsiloxane. The platform allows two-dimentional manipulation of living cells based on the principle of light-induced dieletrophoresis, a process known as OptoElectro Positioning (OEP).[28–30] OEP enables nondestructive maneuvering of individual ASCs into and out of the individual nanopens. Precision microfluidics enables ASC import to and export from the chip; five-color, single-pass fluorescent filter cubes allow multiplexed screening. Beacon has been successfully used for single-cell phenotype and genotype characterization of ovarian cancer patient-derived tumor cells,[31] identification of CRISPR-Cas9–edited primary T cells[32] and for clonal selection of highly productive Chinese hamster ovary (CHO) cell lines.[33] However, to our knowledge, there are no reports of applying the Beacon platform directly for antibody discovery from primary murine ASCs. Therefore, we report here a proof-of-concept experiment of the NanOBlast technique.

Results

Overview of the NanoBlast workflow on Beacon

The fundamental design and features of the Beacon platform, its culturing chip, and OEP technology have been previously described.[32,33] We describe the NanOBlast technique briefly here.

The NanOBlast workflow begins with generating antigen-specific ASCs via in vivo immunization (Figure 1a). ASCs are microfluidically imported into the chip and sequestered into individual nanopens for screening via gravity or OEP. ASCs that secrete antigen-specific IgG are detected using a bead-

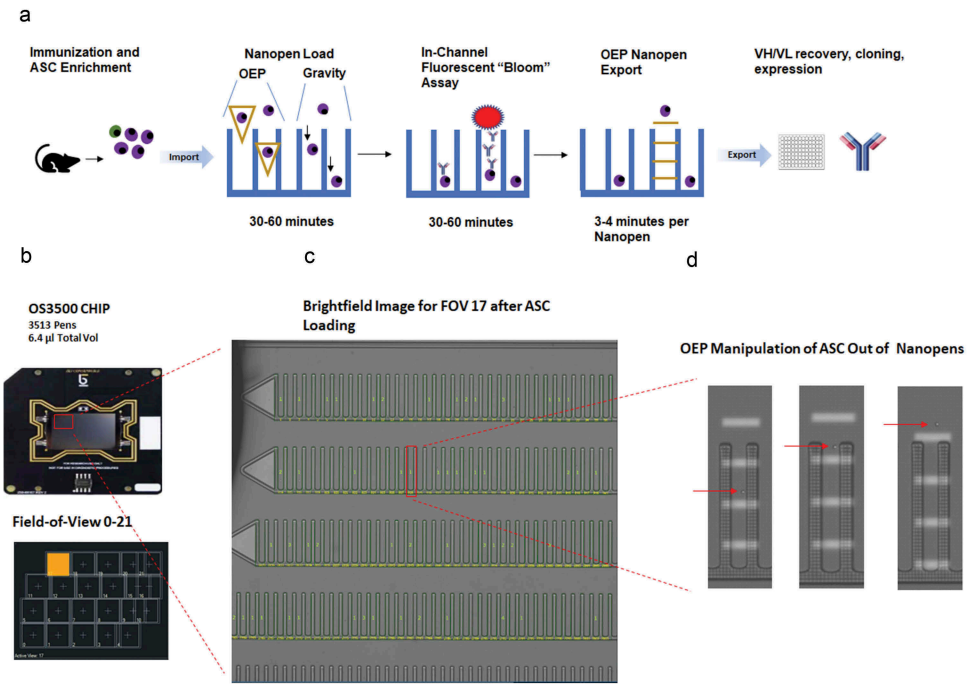

Figure 1. NanOBlast workflow overview. (a). Antigen-experienced immune cells were harvested from wild type mice and enriched using a combination of magnetic negative selection and/or multiparameter FACS sorting. ASCs are loaded into the nanopens of an OptoSelect OS3500 culturing chip via OEP or by inverting the chip and allowing gravity to pull the ASCs downward from the channel into the nanopen. Nanopens are screened for both IgG secretion and antigen specificity using an in-channel multiplex bead-based assay. Immediately after screening, the beads are flushed to waste and the ASC in the selected nanopens is manipulated out of the nanopen using OEP and exported directly into 96-well plates containing lysis buffer using the onboard fluidic system. Single-cell reverse transcriptase (RT) PCR, a modified 5′ RACE amplicon generation step for VH and VL sequence recovery, Golden Gate cloning and recombinant expression using human embryonic kidney 293T cells generate material for binding characterization studies. (b). OS3500 chip from Berkeley Lights containing 3513 polydimethylsiloxane nanopens. The optical train on the Beacon platform subdivides the imaging of the chip into 21 specific FOV. (c). FOV 17 brightfield image showing ASCs sequestered in nanopens after loading overlaid with results of the on-board cell counting algorithm. (d). OEP manipulation of a single ASC out of a nanopen into the channel space of the chip in preparation for export.

based, two-color fluorescent binding assay that produces a characteristic fluorescent bloom. Individual cells of interest are then un-penned using OEP and exported from the chip directly into 96-well plates containing cell lysis buffer. Antibody VH and VL sequences are recovered using single-cell rapid amplification of cDNA ends (RACE), cloned and recombinantly expressed as canonical antibodies using standard methods. The recombinant antibodies are used for binding confirmation and eventual validation in relevant downstream assays. Figure 1b displays the chip and corresponding field of view (FOV) map, Figure 1c is a brightfield image of FOV 17 after loading with ASCs, and Figure 1d demonstrates three sequential images of a single well undergoing OEP export of an individual ASC.

To demonstrate the utility of our NanOBlast antibody discovery workflow, we sought to discover reagent antibodies suitable for bioanalytical assay development in support of therapeutic IgG clinical candidates.

NanoBlast technique proof-of-concept study

Anti-therapeutic IgG ASC enrichment and OptoSelect chip loading

We generated immune animals by challenging CD-1® mice with antigen (i.e., the therapeutic IgG clinical candidate) twice per week over the course of 31 days. We identified animals displaying suitable antigen-specific serum titers using enzyme-linked immunosorbent assay (ELISA). Spleens and lymph nodes (popliteal, inguinal, mesenteric, and brachial) were harvested from the two mice with the highest antigen titer level and processed into single-cell suspensions. ASCs were enriched by a combination of magnetic depletion to remove non-B and surface IgM-expressing B cells and fluorescence-activated cell sorting (FACS) specifically on a B220lo, CD138hi gated population (data not shown). The loss of the B cell marker B220 (also known as CD45R) combined with the high expression of CD138 (Syndecan-1) has traditionally been used to broadly define the antibody-secreting cell population.[34] Enriched ASCs were cultured overnight and imported into an OptoSelect microfluidic chip at a density of 500 cells per microliter. To facilitate expedited screening and minimize the duration cells remained on-chip, the ASCs were penned into individual nanopens using gravity deposition. The chip was then reloaded onto the Beacon platform and imaged to enumerate the overall penning efficiency and the number of cells isolated in each nanopen. In this experiment, 952 cells were isolated into 750 of the available 3513 nanopens (21.3%). Of the 750 loaded nanopens, 615 contained single cells (82%) and 135 contained two or more cells (18%). The penned ASCs were cultured for an additional 75 min by perfusing media through the microfluidic channels before running subsequent anti-idiotypic selective screening assays.

On-chip anti-idiotypic ASC screening

A multiplex, indirect, bead-based competition screening strategy was developed to allow detection of both murine IgG secretion and specific binding reactivity against only the complementarity-determining regions of the human IgG antigen (Figure 2a). A mixture of anti-murine IgG Fc-specific capture beads, an Alexa Fluor 568–conjugated secondary antibody to the murine IgG H + L, and the target antigen linked to Alexa Fluor (AF) 647–conjugated streptavidin was loaded onto the chip in the presence of 10% human serum. Human serum was included to simulate the

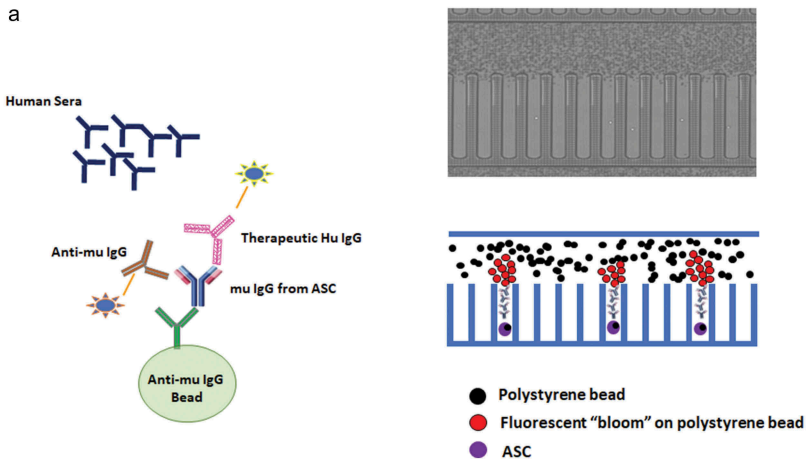

Figure 2. NanOBlast on-chip screening. (a). Cartoon schematic and representative brightfield image of the channel and nanopen space depicting the homogenous bead-based assay design for detection of IgG secretion and antigen specificity in the channel of the OS3500 chip. 3.2-micron polystyrene beads precoated with goat anti-murine IgG, Fc-specific polyclonal antibodies were mixed with AF 568–labelled, goat, anti-murine IgG H+L–specific, polyclonal antibodies; biotin-labelled therapeutic human IgG; streptavidin AF 647; and 10% human serum were imported into the channel space of the chip via onboard fluidics. (b). 45-min (final in the assay series) images of all 21 FOV stitched together depicting the blooms generated for the IgG secretion AF 568 signal as captured in the TRED filter cube of the Beacon. Magnification view of 60 individual nanopens on the chip demonstrating fluorescent bloom formation at the mouth of the nanopen. Time course of bloom formation for nanopen 928 (clone 1A3) demonstrating the change in intensity and size over the duration of the screen. (c). 45-min (final in the assay series) images of all 21 FOV stitched together depicting the blooms generated for the antigen-specific secretion AF 647 signal as captured in the Cy5 filter cube of the Beacon. Magnification view of 60 individual nanopens on the chip demonstrating fluorescent "bloom" formation at the mouth of the nanopen. Time course of bloom formation for nanopen 928 (clone 1A3) demonstrating the change in intensity and size over the duration of the screen. (d). 45-min timepoint images for antigen specificity and IgG secretion of the 13 nanopens from recovered mouse IgGs after constraining the panel to only single exported ASCs.

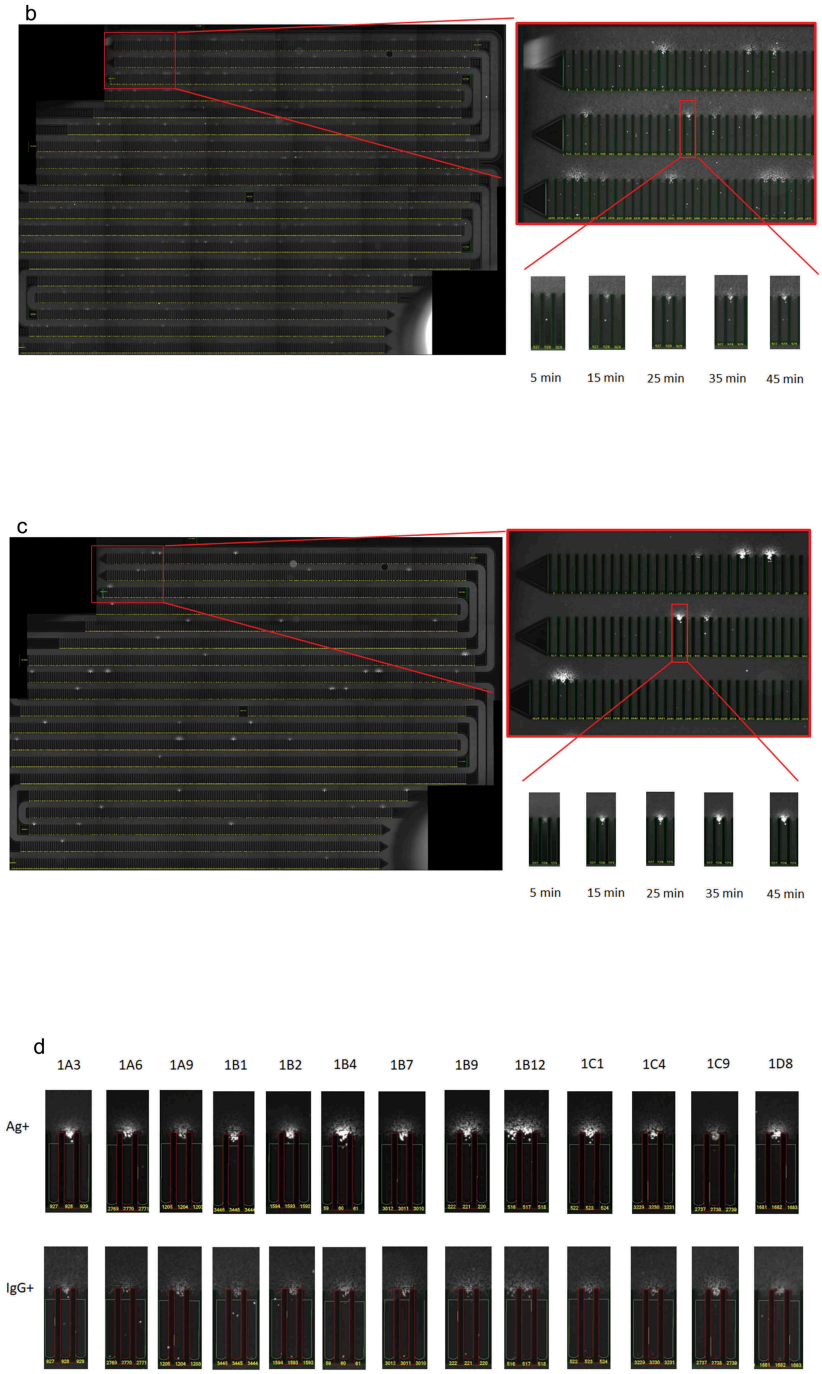

Figure 2. Continued.

complex matrix that will be present in the final LBA, and to ensure that the binding of any mouse antibodies reactive against the constant regions of the therapeutic human IgG would not interfere with the detection of true anti-idiotype antibodies. To identify ASCs producing anti-idiotype antibodies, we imaged each of the 22 FOVs of the chip in both the Cy5 (AF 647) and the TRED (AF 568) fluorophore channels every 5 min over a total duration of 45 min. There were 396 individual images 198 per fluorophore channel) collected over the course of the 45-min assay. Both the TRED and the Cy5 channel images were analyzed using onboard image detection software algorithms and manually verified for the presence of a characteristic fluorescent "bloom" that forms at the mouth of the nanopen (TRED Figure 2b, Cy5 Figure 2c). The presence of anti-idiotypic murine IgG was detected in some nanopens at 5 min, the earliest time point measured (Figure 2c). When the complete chip was analyzed, 201 individual murine IgG-secreting blooms were identified (21% of the total cells penned, Table 1). Importantly, 51 of the IgG-secreting nanopens also displayed reactivity to the therapeutic human antibody (Table 1). Thus, 25% of total IgG-secreting nanopens harbored antibodies that were specific for the antigen and were likely to be therapeutic antibody idiotype–reactive.

Table 1. IgG vs Antigen-specific ASC results per OS3500 Field of View.

Field of View (FOV)	Number of Pens	IgG secreting (AF 568 bloom)	Antigen binding (AF 647 bloom)
0	172	17	5
1	200	9	2
2	200	2	1
3	180	4	2
4	90	8	3
5	192	11	2
6	200	10	3
7	196	13	2
8	200	8	1
9	155	5	1
10	83	2	0
11	160	12	5
12	200	14	2
13	200	11	3
14	200	13	4
15	145	2	2
16	73	12	1
17	151	19	7
18	160	9	1
19	160	6	2
20	132	10	1
21	64	4	1
All	3513	201	51

VH and VL recovery, cloning, and recombinantexpression

To recover the potential anti-idiotype antibodies identified using NanOBlast, the individual cells located within 44 of the 51 antigen-specific nanopens were unpenned using OEP and exported directly into a 96-well plate containing cell lysis buffer. The antigen-specific ASCs in the remaining seven nanopens were not exported because the bloom intensity of the antigen-specific signal, although detectable, was relatively weak compared to other nanopens (data not shown). To confirm successful export, brightfield image analysis of the channel space immediately after unpenning, but directly before export, was performed (data not shown). Figure 2d displays IgG secretion and antigen-specific bloom images for 13 representative nanopens visually confirmed to contain single ASCs.

A total of 30 paired VH and VL sequences from the 44 exported ASCs were successfully recovered. We constrained our analysis, and subsequent efforts, to focus only on nanopens containing single cells to eliminate the recovery of noncognate VH and VL pairs caused by multiple ASCs within a single nanopen. Comparison of the recovered sequences indicated 13 unique antibody sequences that arose from at least 10 different V-(D)-J recombination events (Figure 3a; Table 2).

Characterization of rescued antibodies and selection of tool reagents for the therapeutic bioanalytical method

The 13 recovered sequences were cloned into an expression vector using a murine IgG2a isotype, transiently transfected into human embryonic kidney 293T cells and the antibody-containing, conditioned media was harvested 5 days later. The supernatants were quantified and displayed a range of concentrations (2.3–117 µg/ml (Table 1)), typical of murine IgGs.[26]

Normalized antibody samples were assayed for antigen-specific binding using an IgG-capture ELISA, like that used in the on-chip, bead-based NanOBlast strategy (Figure 3b). To differentiate the antibodies based on their relative binding strength and to ensure the relative binding affinity was strong enough for downstream LBA development, the antigen concentration was titrated from 6.6 nM to 66 pM. Under these limited antigen conditions, all 13 recombinant IgGs bound antigen in a dose-responsive manner (Figure 3c).

To select antibodies appropriate for bioanalytical method development, the recombinant murine antibodies were purified and biotinylated. For this bioanalytical assay, a pair of unique and complementary anti-idiotype antibodies are required to enable simultaneous capture and detection of the fully human therapeutic in the presence of human IgG-containing serum. Iterative combinations of each of the NanOBlast-derived antibodies, as either capture or biotin-detection reagents, were assayed via sandwich ELISA (Figure 3d). Under these conditions, antibody 1A3 was observed to be the most appropriate capture reagent as it showed no serum matrix effects and permitted a highly sensitive LBA construction with the flexibility of using multiple detection antibodies. When paired with antibodies 1B4, 1B9, or 1C1, antigen capture via 1A3 enabled the construction of a specific LBA with a lower limit of quantitation below 1 ng/ml (Figure 3e), thereby establishing a robust LBA easily capable of quantifying the free therapeutic IgG levels expected in first-in-human clinical studies.

Discussion

Although the NanOBlast workflow described here shares some similarities with previously described single-cell ASC discovery methods,[13–22] it introduces an entirely novel nanofluidic culture and screening paradigm using the Beacon platform. To our knowledge, Beacon is the only tool that enables massively parallel, precise, digitally driven control over the import, culture, screening, analysis, and export of non-immortalized primary antigen-specific ASCs. The ability to rapidly sequester and culture single, primary ASCs in individual, software-tracked nanopens; screen the secreted antibodies for desired phenotypes; and digitally document the entire workflow enables unprecedented speed and utility for commercial antibody discovery.

While dependent on the immunogenicity of the antigen and the exact conditions of the immunization campaign, antigen-specific ASCs in wild-type hyperimmunized mice are typically present at frequencies <0.1% (Ref. 29, Amgen unpublished data). As such, enrichment of immune cells for relevant ASCs is necessary prior to initiate NanOBlast to efficiently use the 3513 nanopens of an OS3500 chip. Applying both magnetic negative selection and a multiparameter FACS sort to primary mouse lymphocytes enabled us to reduce the initial ~10^8 mixed population of cells to ~10^4 ASCs with the desired characteristics. The current NanOBlast workflow would not be possible using an unenriched population of lymphocytes due to the low frequency of ASCs amongst primary lymphocytes and the fixed number of nanopens on a chip (3513). Increasing the antigen-specific ASC enrichment effectiveness before chip loading is of obvious value in terms of efficiency and recoverable antibody panel size. Specific immunization and enrichment strategies will need to be developed for the particular in vivo model or antibody generation platform, as well as for the specific B cell compartment targeted. A successful strategy must balance ASC generation, enrichment (e.g., purity, yield) and cellular viability such that they can be manipulated and screened on Beacon.

Loading single cells into individual nanopens is a critical requirement of our NanOBlast workflow. A nanopen containing more than 1 ASC potentially complicates the interpretation

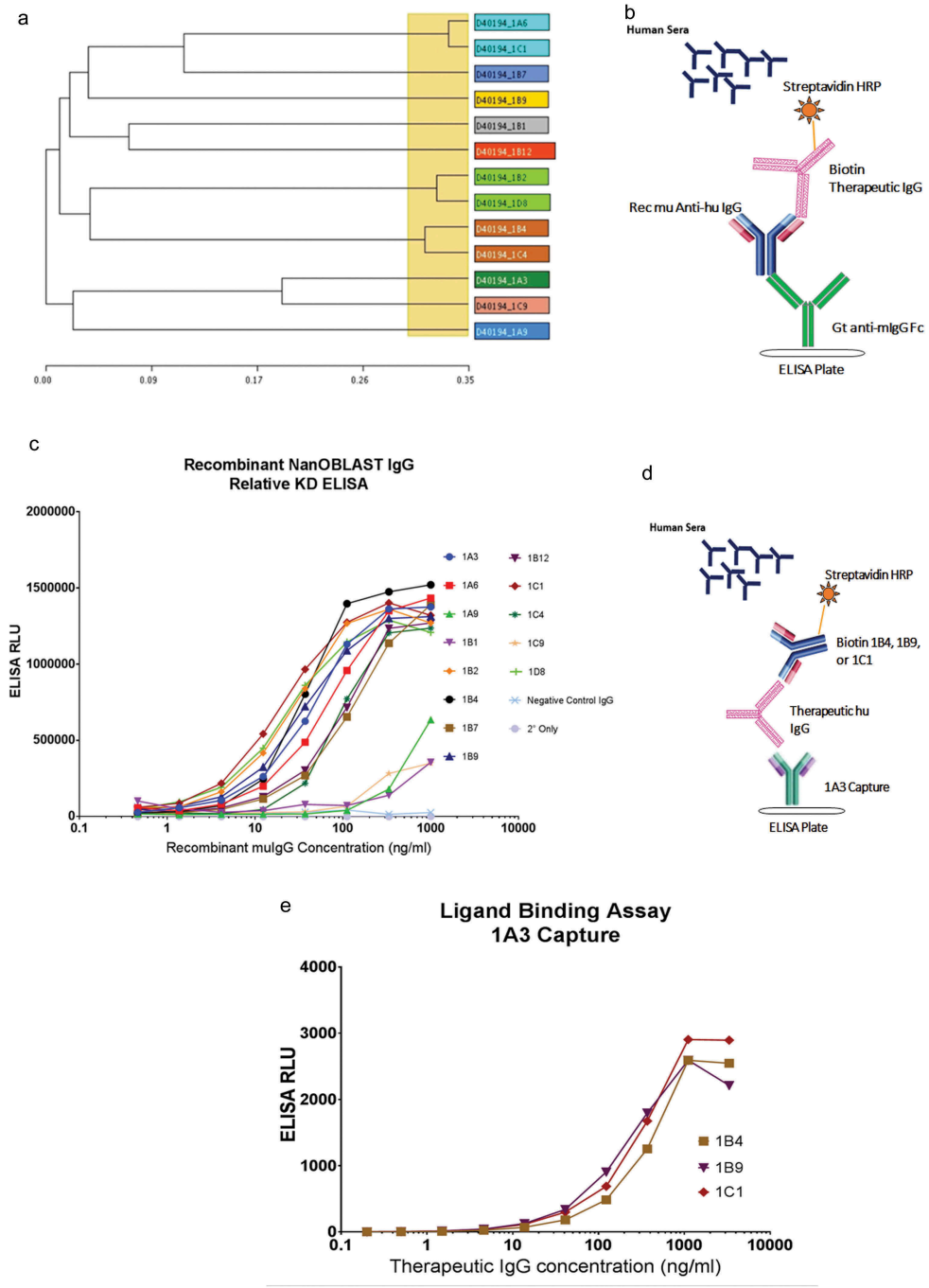

Figure 3. Recombinant Mouse IgG Characterization. (a). VH and VL V(D)J dendrogram illustrating the sequence diversity of the single-cell, recovered, antigen-specific clones. The yellow box indicates a chosen maximum distance between two antibodies to allow grouping and is based on a calculation of similarity equal to the number of mismatches divided by the sequence length. The tree length is on the x-axis and antibodies with a distance less than 0.1 from each other were grouped together resulting in 10 separate antibody clades. (b). Plate-based immunoassay design for verification of antigen specificity and relative affinity of the recombinantly expressed antitherapeutic murine IgGs. (c). Dose response, antigen-specific immunoassay binding curves using a limiting concentration of 66 pM of the therapeutic IgG. (d). Design of the completed LBA for evaluation of free therapeutic IgG in human serum. E. LBA with lower limit of quantification < 1ng/ml using 1A3 as the capture antibody on the plate and either 1B4, 1B9, or 1C1 as the detection antibody in a matrix of human serum.

of fluorescent blooms because the signal may be generated by more than one specific antibody. It can also confound the downstream analysis if noncognate pairs of VH and VL are combined for recombinant expression. The efficiency of loading single cells in the nanopens is directly related to the density of the ASCs imported into the channel of the chip and the percentage of clumped cells in the preparation. Software-driven OEP nanopen loading offers enhanced precision compared to gravity loading due to the image detection and targeting control that the Berkeley Lights Cell Analysis Suite (CAS) version 1.3 software provides. We have successfully performed this semi-automated loading strategy on ASCs harvested from wild-type mice, transgenic mice (e.g., XenoMouse®), and hybridomas (unpublished data). However, optimization of the exact voltage and frequency of the current applied to the chip, duration of application, and temperature of the chip are necessary to

Table 2. Anti-Idiotypic IgG Recovery and Expression.

Molecular Recovery of Antibody[a]						
Pen ID	Export plate well	VH germline	% Identify	VL germline	% Identify	Recombinant IgG μg/mL[b]
928	1A3	VK1|1–110*01/JK4|4*01	98.8	VH1|1–82*01/D2|2–11*01|RF1/JH3|3*01	94.3	16.6
2770	1A6	VK6|6–13*01/JK2|2*01	92.6	VH1|1–14*01/D4|4–1*02|RF3/JH4|4*01	98.9	4.7
1204	1A9	VK8|8–21*01/JK5|5*01	97.5	VH5|5–6*01/D4|4–1*02|RF3/JH2|2*01	95.4	2.3
3445	1B1	VK6|6–23*01/JK4|4*01	97.5	VH2|2–6-1*01/D4|4–1*02|RF1/JH4|4*01	95.4	117.1
1593	1B2	VK6|6–17*01/JK1|1*01	97.5	VH4|4–1*01/D4|4–1*01|RF3/JH2|2*01	96.6	37.4
60	1B4	VK5|5–43*01/JK4|4*01	96.3	VH9|9–3*01/D4|4–1*01|RF1/JH2|2*01	98.9	14.1
3011	1B7	VK4|4–68*01/JK5|5*01	98.8	VH1|1–14*01/D2|2–2*01|RF2/JH2|2*01	95.4	2.4
221	1B9	VK4|4–74*01/JK2|2*01	100.0	VH1|1–34*02/D4|4–1*02|RF3/JH4|4*01	95.4	5.1
517	1B12	VK4|4–91*01/JK2|2*01	97.5	VH2|2–9-1*01/D4|4–1*01|RF1/JH4|4*01	96.6	40.5
523	1C1	VK6|6–13*01/JK2|2*01	93.8	VH1|1–14*01/D4|4–1*02|RF3/JH4|4*01	98.9	43.9
3230	1C4	VK5|5–43*01/JK4|4*01	96.3	VH9|9–3*01/D4|4–1*01|RF1/JH2|2*01	93.1	12.5
2738	1C9	VK1|1–110*01/JK2|2*01	100.0	VH1|1–61*01/D4|4–1*01|RF3/JH3|3*01	93.1	4.6
1682	1D8	VK6|6–17*01/JK1|1*01	98.8	VH4|4–1*01/D4|4–1*01|RF3/JH2|2*01	96.6	40.4

[a]Germline analysis based on IMGT database. [b]Quantification of IgG in HEK293T supernatants determined by Forte Bio.

effectively use OEP manipulation without damaging fragile ASCs. The precise combination of optimal conditions will likely depend on the harvested B cell compartment, enrichment processing, and culture media formulations. Optimizing semi-automated OEP is the subject of continued experimentation in our lab. Moreover, primary murine ASCs frequently aggregate in ex vivo culture (data not shown), and we speculate that the use of deoxyribonuclease or other de-clumping reagents may encourage ASCs to remain as singlets during pre-chip processing. More homogenous single-cell suspensions will undoubtedly enable more efficient single-cell nanopen loading for both OEP and gravity methodologies.

The strategies underpinning our NanOBlast on-chip screening assays were threefold: 1) identify nanopens containing ASCs secreting antibodies of the IgG isotype, 2) multiplex the assays to reduce overall on-chip processing time, and 3) identify ASCs secreting anti-idiotypic (not framework-specific) antibodies by executing the bead-based assay in the presence of 10% human sera. By performing the assay in an excess of unlabeled human IgG along with the labeled target antigen, a competitive binding environment was established. Nanopens containing ASCs producing antibodies specific for common epitopes present on human antibodies (e.g., constant domains) were unable to bind to the target antigen and therefore could not form fluorescent immune complexes (i.e., blooms). This assay format allowed us to identify ASCs secreting antibodies that were likely to be anti-idiotypic toward our therapeutic IgG antigen.

Quantitative ranking of the binding of antibodies is frequently used to characterize candidate molecules and can be accomplished using ELISA, FACS, surface plasmon resonance systems (e.g., Biacore), and other methodologies.[7,8,11,15,26,35–40] As of early 2019, the Beacon software was unable to generate quantitative values for fluorescent blooms. Despite the high-content imaging capabilities, this limited the built-in analysis to a binary (yes/no) result. The bloom size, intensity and rate of change resulting from the accumulation of antibody/antigen complexes are likely governed by multiple factors, including assay design, IgG secretion rate, actual location of the ASC within the pen, and the binding kinetics (i.e., determination of association [k_{on}] and dissociation [k_{off}]) of the IgG molecule to its cognate antigen. This blend of variables (known and unknown), makes it challenging to determine the role that affinity of the ASC-derived IgG plays in bloom formation. While it is tempting to analyze the bloom images and extrapolate relative rankings of nanopens based on factors such as bloom intensity and size, we do not yet have data that validates this approach. The application of advanced machine learning algorithms, combined with training data sets of verified recombinant NanOBlast IgGs, may be necessary to confidently enable automated ranking on the Beacon™ platform.

Although the goal of this study was not to make direct quantitative comparisons to other antibody discovery methods, we can speculate how NanOBlast might compare to traditional hybridoma generation workflows, specifically in terms of total quantity and diversity of recovered antibody sequences. In 1989, Schmitt et. al described an optimized hybridoma electrofusion protocol capable of generating one hybridoma from 5000 input splenic lymphocytes.[12] Even if we assume that hybridoma process optimization over the last 30 years resulted in protocols with 50X greater hybridoma generation efficiency (e.g., one hybridoma for every 100 input ASCs), the 615 single ASCs identified here would have translated to only six IgG secreting hybridomas. Using data generated in this study (Table 1) as a guide, 25% of these recovered hybridomas would secrete antigen-specific antibodies, resulting in a total of 1–2 hybridomas available for further analysis. The 615 single-penned ASCs analyzed in our NanOBLast workflow represented less than 10% of the total ASCs recovered from our FACS-based enrichment process, yet we still recovered 13 unique antibody sequences representing 10 distinct clades. We feel confident that as we advance the NanOBLast workflow in terms of enrichment efficiency and screening scale, we will be able to sample increasingly larger portions of the immune repertoire of hyperimmunized animal models with an efficiency that is simply not possible with even the most highly optimized hybridoma generation methods.

Taken together, our data indicate that NanOBlast is a rapid and effective method for isolating, characterizing, and recombinantly producing antibodies in less than 60 days directly from antigen-experienced murine ASCs. Although indispensable for the successful development of protein therapeutics, our focus on identification of tool reagents from wild-type mice represents a jumping-off point for antibody discovery from in vivo-derived ASCs using this technique. Therapeutic antibody discovery leveraging transgenic mouse platforms, such as XenoMouse®,[41] would be of great value, as would

the direct characterization and recovery of human ASCs harvested from the peripheral blood of patients undergoing active disease. Indeed, the species-agnostic nature of NanOBlast opens the door to de novo antibody discovery from any species where antigen-experienced ASCs can be identified and enriched. Expanding the library of reliable, effective and high-content on-chip assays (e.g., receptor-ligand blocking, antibody competition, epitope binning, affinity assays) using the nanopen architecture and the five-color imaging capability of the Beacon platform will be a necessary next step to realizing the full potential of NanOBlast. In this regard, we have already made significant progress using antigen-expressing cells in place of the soluble antigen-bead system described here. This raises the possibility of more physiologically relevant screening scenarios for therapeutically interesting complex membrane targets, as well as functional, reporter-style, cell-dependent assays. Concurrent with expansion of the operational assay catalogue, ongoing platform and software improvements for the Beacon platform achieved by Berkeley Lights continue to increase tool functionality and quantitative ranking of nanopen assays. Moreover, we feel the overall workflow timeline can be executed even more rapidly. Successful VH and VL recovery and recombinant expression in 5–7 days have been demonstrated,[35,37,38] as have rapid immunization strategies.[42,43] Combining approaches such as these with the 1-day screening process of the NanOBlast method described here, complete antibody discovery workflows using antigen-experienced, affinity-matured ASCs could be executed in as little as 3–4 weeks. We anticipate that this novel methodology will ultimately advance the discovery of critically important tool and therapeutic antibodies, better enabling the treatment of human diseases.

Materials and methods

Antigen supply and immunization

A human IgG1 mAb clinical candidate that targets a cell membrane antigen was used as the antigen in this study. This mAb was generated by gene synthesis and recombinant expression in CHO host cells. It was recovered from the clarified CHO cell condition media using a three-step process including 1) affinity capture using MabSelect SuRe (GE Healthcare Life Sciences), 2) purification on an SP HP column (GE Healthcare Life Sciences), and 3) dialysis into sodium acetate buffer (A52Su: 10 mM acetic acid, 9% sucrose, pH 5.2) for long-term stability.

Mice were housed in groups at an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facility. Animals were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals*, 8th Edition. All research protocols were reviewed and approved by the Amgen Institutional Animal Care and Use Committee. Ten CD-1 mice (Charles River Laboratories) were immunized with antigen a total of 10 times over 31 days. Boost 1 consisted of 5 µg of antigen emulsified in 100 µL Complete Freund's Adjuvant (Sigma Aldrich) delivered to four spots subcutaneously. Boosts 2–10 were delivered every 3 to 4 days and consisted of 5 µg antigen emulsified into 300 µL Sigma Adjuvant System (Sigma Aldrich) delivered subcutaneously to six spots alternately each time between dorsal and ventral sides of the mouse. Submandibular check bleeds of 150 µL were taken on day 25 for serum titer analysis. Mice 8, 9, and 10 were boosted intraperitoneally on day 27 with 50 µg of the human IgG antigen resuspended in Dulbecco's phosphate-buffered saline (PBS) (Gibco Life Sciences). On day 31, mice 8 and 9 were euthanized, and spleens and LNs harvested for B cell processing. The remaining eight mice not harvested were euthanized immediately after the development of the LBA was validated.

Immunogen biochemical conjugation

Biotinylated forms of the therapeutic IgG were generated by buffer-exchanging the human IgG into PBS using Zeba columns (Thermo Fisher Scientific), and then adding a 10-fold molar excess of NHS-PEG-Biotin (Thermo Fisher Scientific) to the antigen. After 60 min incubation at RT, excess biotin was removed again using a Zeba column and the concentration confirmed via A280. The horseradish peroxidase (HRP) form of the antigen was generated by adding a 10-fold molar excess of SAT-PEO4 (Thermo Fisher Scientific) to the antigen in Dulbecco's PBS and incubating for 60 min at room temperature. 50 mM hydroxylamine-HCl was added to the preparation and incubated for 90 min again at room temperature. Excess hydroxylamine-HCL and SAT-POE4 were removed using Zeba column. A 4-fold molar excess of maleimide-activated HRP (Thermo Fisher Scientific) was added to the preperation and incubated for 60min at RT. The excess unreacted malemide HRP was not removed and the final concentration of the conjugate was estimated assuming 90% recovery of the starting concentration of human IgG.

ELISA serum characterization

384-well plates were coated with 2 µg/mL of a fibrinogen-streptavidin dextran conjugate in PBS overnight at 4°C. Plates were washed with PBS plus 0.05% Tween20 (Sigma) and blocked with 50 µL per well of Superblock (Thermo Fisher Scientific). 20µL per well of a mixture of 250 ng/mL each of biotin- and HRP-conjugated therapeutic antibody diluted in Superblock buffer containing 20% human serum (Calbiochem) was added to each well. 20 µL per well of serially diluted murine antiserum was then added to the plate and incubated overnight. The following morning, plates were washed 4 times and 25 µL per well of Femto substrate (Thermo Fisher Scientific) was added to each well. The plate was read on a Spectramax M5 (Molecular Devices) reader using 25 ms integration time.

ASC harvest and enrichment

Selected mice were boosted by injecting 150 µL PBS (Gibco) containing 50 µg of therapeutic IgG antigen intraperitoneally. Four days after boost, spleen and mesenteric, popliteal, inguinal, and brachial lymph nodes were harvested from each animal. The spleen and lymph nodes were then processed into a single cell suspension using a GentleMACS cell dissociator (Miltenyi Biotech). The cell suspension was washed twice in PBS plus 2% fetal bovine serum (FBS) before red blood cell lysis with 8.3 g/L ammonium chloride in 10 mM

trisaminomethane-HCL, pH 7.2 for 5 min at room temperature. Non-B cells and IgM-expressing B cells were removed using an EasySep Mouse Pan B Isolation kit (StemCell) following the manufacturer's protocol with a slight modification. Briefly, a biotinylated rat anti-IgM mAb (Amgen) was added to the Pan B isolation antibody cocktail mixture step at a final concentration of 5 μg/mL. The isolated B cells were then counted and stained for sorting. The B cells were blocked with Mouse BD Fc Block™ purified anti-mouse CD16/CD32 mAb 2.4G2 (BD Bioscience) for 5 min at 4°C. The cells were incubated with APC-H7 Rat Anti-Mouse B220 (clone RA3-6B2, BD Bioscience) and PE Rat Anti-Mouse CD138 (clone 281–2, BD Bioscience) for 30 min at 4°C. The samples were washed with PBS plus 2% FBS twice and incubated with 7AAD (BD Bioscience) for 10 min on ice. All antibodies were used at a final concentration of 1 μg of antibody per 1 million cells. The B220low, CD138Hi population was sorted on a BD FACS Melody (BD Bioscience) using the manufacturer's recommended settings. The sorted cells were incubated in a 37°C incubator with 5% CO_2 until needed.

Beacon ASC import, assay, and export

OptoSelect 3500 chip was loaded onto Beacon, and the wet chip Python script was executed using the one-step priming reagent (Berkeley Lights Inc.) and 0.1% Pluronic F127 rinse (Invitrogen). The fluidics were primed with cell culture media consisting of Iscove's Modified Dulbecco's Media basal media (Gibco Life Technologies), 15% low IgG FBS (Gibco), 10mM HEPES (Gibco), one part NEAA (Gibco), 55uM 2-mercaptoethanol (Gibco), one part Penicillin-Streptomycin-Glutamine (Thermo Fisher Scientific), and a proprietary blend of additional supplements designed to enhance ex-vivo ASC survival.

Enriched ASCs were imported using the small volume import script with 0μL leading volume, 6 μL package volume, and 10μL lagging volume. Cells were cultured at 36°C during the duration on the chip. The antigen-screening mixture consisted of 900 μL of growth media, 100μL of human serum, 1 μL of goat anti-mouse IgG H + L AF 568 (Invitrogen), 1μg/mL of biotinylated therapeutic IgG, and 500 ng/mL of Streptavidin AF 647 (Invitrogen). 300μL of 3.2 μM polystyrene beads coated with a goat polyclonal antibody specific to the Fc regions of mouse IgG (Spherotech #MPFc-30–5) were rinsed 1 time in PBS and resuspended in 30μL of the antigen screening mixture. The beads were resuspended and 20 μL of the mixture was imported directly into the chip at 1 μL/sec using the manual Nest pump functions of CAS.

The screening assay was initiated using the multiplex assay script on the CAS using channel priority with image capture iterations every 5 min for a total of 10 iterations. The Texas Red channel for the AF 568 fluorophore was set to 1500 ms exposure time, and the CY5 channel for the AF 647 fluorophore was set to 2000 ms. The freeze valve was turned on during the entire length of the assay. Immediately following assay completion, the chip was flushed 3 times with culture media to remove beads from the fluid path. Fluidic lines were flushed further by removing the OS3500 chip from the nest, pumping 10 cycles of deionized water through the lines of Beacon, reloading a flush chip and priming the lines once again with culture media 4 times. The OS3500 chip containing the ASCs was reloaded on the nest, recalibrated in the CAS and flushed another 3 times with culture media in preparation for export. A 96-well forensic grade PCR plate (Eppendorf) was prepared to receive nanopen exports by adding 5 μL of TCL buffer (Qiagen) supplemented with 5 mM dithiothreitol (Thermo Fisher Scientific) to each well. 15 μL of mineral oil (Sigma) was finally added to the top of each well, and the entire plate was centrifuged for 1 min at 600 g. The plate was loaded in the incubator WP1 of Beacon, and the small volume PCR export script was executed using manually imported nanopen IDs. OEP was set to 4.2v and 1200kHz, the temperature of the chip was reduced to 18°C and 5 μL of total volume containing the nanopen contents was exported using the automated script. The plate was immediately frozen at −80c after all exports were completed.

Molecular recovery

RNA purification and reverse transcriptase (RT) PCR were performed using a modified protocol provided by Berkeley Lights Inc. RNA was purified using AMPure RNA Clean XP kit and eluted directly into a 9 μL RT reaction with Maxima RNaseH minus RT. A dT primer with adaptor (P1) and a 5′ template switching primer (P2) was used in the RT reaction. The RT reaction was incubated at 42°C for 90 min, followed by 10 cycles of 50°C for 2 min followed by 42°C for 2 min, then heat-inactivated at 75°C for 15 min and left at 4°C until the next step. The 9 μL RT reaction product was added to a PCR mix using KAPA HiFi HotStart Readymix with primer P3 in a reaction, amplifying the complementary DNA (cDNA) in 30 μL total. The cDNA amplification was performed at 98°C for 3 min, followed by 20 cycles of 98°C for 15 s, 65°C for 30 s, 72°C for 6 min, with a final incubation at 72°C for 10 min, and left at 4°C until the next step. A 1 μL portion of the cDNA amplification product was used to amplify the gamma and kappa chains in separate 15 μL reactions with primers in the antibody constant regions (MoG_AS and MoK_AS) and P4 to obtain the specific product for sequencing. PCR conditions were 98°C for 3 min, followed by five cycles of 98°C for 20 s – 65°C for 45 s – 72°C for 45 s, 10 cycles of 98°C for 20 s – 60°C for 45 s – 72°C for 45 s, 10 cycles of 98°C for 20 s – 58°C for 45 s – 72°C for 45 s and final extension of 5 min at 72°C. The specific PCR products were sequenced at Genewiz with the corresponding antibody constant region primers (MoG or MoK). Sequence analysis was performed using in-house software and aligned to the international ImMunoGeneTics information system® (http://www.imgt.org) database for germline determination.

Cloning and recombinant antibody expression

Sequences from the unique variable regions were synthesized with adaptors for cloning at Integrated DNA. Cloning was performed using BsmBI restriction sites into in-house vectors for expression. Transfection into human embryonic kidney 293T cells (CRL-3216,™ ATCC) was performed using 293Fectin according to manufacturer's protocols, modified to include 1 μL of 293Fectin per 1 μg of DNA in total precomplex volume of 100 μL. Supernatants were harvested

5 days after transfection and quantified via Forte Bio Octet (Molecular Devices) using anti-moFv AMQ sensors and a murine IgG2a isotype standard curve.

Relative affinity ELISA of recombinant anti-idiotypic IgGs

384-well plates were coated with 2 µg/mL of goat anti-murine IgG,-Fc specific polyclonal antibody (Thermo Fisher Scientific Cat # 31170) in PBS overnight at 4°C. Plates were washed with PBS plus 0.05% Tween20 (Sigma) and blocked with 50 µL per well of Superblock (Thermo Fisher Scientific). 20 µL per well of serially diluted recombinant antitherapeutic IgGs were added to the plates and incubated for 60 min at room temperature. Plates were washed 2 times and 20 µL per well of a 10 ng/mL solution of HRP labelled therapeutic IgG suspended in 20% human serum (Calbiochem) and Superblock (Thermo Fisher Scientific) was added to the plate. After a 60-min incubation, plates were washed 4X and 25 µL/well of Femto substrate (Thermo Fisher Scientific) was added to each well. The plate was read on a Spectramax M5 (Molecular Devices) reader using 25 ms integration time.

Bioanalytical LBA screening

The anti-IgG therapeutic antibodies were purified by protein G affinity purification (GE Healthcare) from 50 mL conditioned media and eluted by 0.5 M acetic acid, and then neutralized immediately with 2 M phosphate buffer with pH of 8.2. The concentration of the antibodies was measured by spectrophotometer at 280 nm.

The purified antibodies were screened in combinatorial approach as previously described.[8] Briefly, an ELISA plate (Corning 3690) was coated with antitherapeutic antibodies at 1 µg/mL in one part PBS and incubated overnight at 4°C. The ELISA plates were washed and blocked with I-Block™ (Applied Biosystems) buffer (one part PBS plus 0.2% I-Block and 0.05% Tween20) for 1 h at room temperature before use. Standards were prepared by spiking the therapeutic antibody into 100% serum from different species (mouse, rat, cynomolgus monkey, and human). The prepared standards samples were then diluted 1:10 (or 1:4 for high sensitivity assay) in the assay buffer (I-Block with 5% BSA). Then, 50 µL of diluted standards samples were loaded into each well of the ELISA plate coated with antitherapeutic antibodies and allowed to incubate for 1.5 h at room temperature. After a wash step, 50 µl of biotin conjugated of antitherapeutic antibodies at a concentration of 100 ng/mL in the assay buffer was added to each well of the plate and incubated for 1.5 hr. After a wash step, 50 µl of streptavidin conjugated to horseradish peroxidase at 200 ng/mL in the assay buffer was added to each well of the plate and incubated for 15 min. After a final wash step, a tetramethylbenzidine peroxidase substrate solution (KPL Inc) was added to each well and absorbance of the developed color was measured by kinetic read at 650 nm by a colorimetric plate reader SpectraMax (Molecular Devices).

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