Accelerated Discovery of Unique Anti-PD-L1 Antibodies from Spleen Versus Bone Marrow of Immunized Mice by Single Plasma B cell Cloning on the Beacon® Platform

Ye Jin[1], Dandan Lv[2], Lina Xu[2], Rui Wang[2], Shaoping Hu[2], Vincent Pai[3], Minha Park[3], Ravi Ramenani[3], Maryam Shansab[3], Po-Yuan Tung[3], Amanda Goodsell[3], Adrienne Higa[3], Shireen Khan[1]

[1]ChemPartner San Francisco, South San Francisco, CA; [2]Shanghai ChemPartner Co., Ltd., Shanghai, China; [3]Berkeley Lights, Inc., Emeryville, CA

ChemPartner — Dedicated to LifeScience

BERKELEY LIGHTS

ABSTRACT

PD-L1 is a key inhibitor of T cell activation that is often over-expressed in cancer to escape immune surveillance and promote tumor progression. Blocking antibodies against PD-L1 or its receptor, PD-1, have shown significant clinical benefit in some patients with PD-L1 expressing tumors. Hence, there is great interest in generating therapeutic antibodies against these targets to counteract the immune suppression mechanism that tumors rely on for survival. Most of the anti-PD-L1 therapies in the clinic have been generated by standard hybridoma technology but it remains to be determined whether superior antibodies with greater diversity, affinity, and/or functional activity could be generated using single B cell cloning. Using the Beacon® platform (Berkeley Lights, Inc., (BLI)), we have screened tens of thousands of primary single plasma B cells to identify unique antibodies against PD-L1. We have immunized Balb/c mice with recombinant Fc fusion of human PD-L1 extracellular domain (ECD) and isolated CD138+ plasma B cells from bone marrow and spleen from an 8 week immunization. Plasma B cells from both spleen and bone marrow were penned as single cells onto OptoSelect™ chips for analysis. A series of assays were performed in tandem including bead-based binding/blocking assays, cell-based binding to CHOK1 cells engineered to overexpress human PD-L1, and cell-based blocking assays using PD-1. This series of assays on the Beacon enabled identification of roughly 300 antibodies binding to PD-L1, some of which blocked binding of PD-1 to PD-L1 on cells. Interestingly, a majority of PD-L1 specific antibodies were identified from plasma B cells isolated from bone marrow even though the yield of plasma B cells from bone marrow was less than 20% of what was obtained from the spleen. Single plasma B cells were exported by the Beacon platform for antibody sequence recovery. After reverse-transcription, amplification, and single plasma B cell sequencing by NGS, we evaluated the sequence diversity of anti-PD-L1 antibody hits. Taken together, our results from plasma B cell cloning on the Beacon show that plasma B cells secreting functional antibody candidates can be identified within one week compared to 1-2 months for the standard hybridoma campaign, thus substantially accelerating the antibody discovery process. In addition, this technology could enable rapid comparison of immunization strategies, mouse strains, and lymphoid organs from which to isolate B cells to maximize recovery of target specific functional antibodies representing the diversity of the immune repertoire.

MATERIALS AND METHODS

Immunizations:

Balb/c mice at 6-10 weeks old were immunized at Pacific Biolabs (Hercules, CA) by interperitoneal injection with recombinant human PD-L1 ECD protein at 50 µg/animal with CFA. After 14 and 35 days, animals received 25 µg of human PD-L1 ECD with IFA and, on day 56, animals received a final boost of 25 µg/animal with saline. Test bleeds were taken at day 21 and day 42 and titers were measured by ELISA. Titer analysis showed increasing titers between the first and second boost. At day 57, spleen and bone marrow were harvested from immunized animals for B cell isolations. Two mice were immunized per group and three groups total were immunized at two week intervals to allow time between studies for data analysis. A total of three Beacon experiments were completed using bone marrow and spleen from immunized animals.

B cell Isolations:

Single cell suspensions were prepared from bone marrow and spleen harvested from two immunized mice per round by dissociating tissue and filtering through a 40 µm cell strainer. Spleen cells were overlayed onto 15 mL Ficoll to remove red blood cells. Cells from bone marrow or spleen were counted and kept separate throughout the isolation procedure. B cells were isolated using the CD138+ mouse plasma cell isolation kit (Miltenyi Biotec, Cat. No. 130-092-530) according to manufacturer's recommendations. Briefly, non-plasma cells were depleted using the non-plasma cell depletion cocktail and added to the LD column. Cells that did not bind to the LD column were washed and CD138 microbeads were added to positively select CD138+ cells which were captured on the MS columns. At the end of the isolation procedure, plasma B cell yield ranged from 20,000 – 50,000 cells from bone marrow and 40,000 – 150,000 from spleen.

FACS Staining:

Aliquoted cells were pelleted in a 96 well plate and washed one time with FACS buffer (1xPBS, 2%BSA, 5mM EDTA). Cells were blocked with Fc block (Biolegend Clone 93, Cat. No. 101319) for 15 minutes at 4°C. After wash, the antibody cocktail was added and resuspended cells were incubated for 30 minutes at 4°C in the dark. After a final wash, the samples were resuspended and acquired on a FACSCelesta (BD Biosciences, San Jose, CA). Data was analyzed with Flowjo software (Flowjo, Ashland, OR).

PCR Amplification and Sequencing:

Following export from the Beacon platform, paired heavy chain and light chain (VH/VL) sequences were recovered for single B cells using BLI's proprietary protocols. The protocols end with amplified whole mRNA, which can be further used towards targeted amplification or direct cloning workflows. For these experiments, templates recovered from the single B cells were barcoded, pooled, and sequenced using a MiSeq (Illumina, Inc., San Diego, CA). Paired VH/VL sequences were recovered using a BLI custom bioinformatics pipeline.

INTRODUCITON TO BLI TECHNOLOGY

Image 1. Beacon® Platform

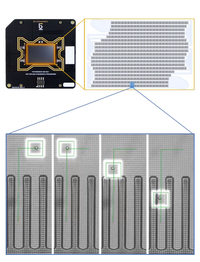


Image 2. OptoSelect™ Chip
Light manipulation + microfuidics
Single Cell Isolation in NanoPens™
Thousands in parallel

Figure 1.
(A) The Beacon platform blends novel nanofluidic design with semiconductor technology to enable tens of thousands of single cell experiments in parallel. (B) BLI's OptoSelect chip overlays a perfusion-based nanofluidic system over light-activated phototransistor arrays. This enables the rapid and precise positioning of single cells or other micro-objects using controlled patterns of light. The OptoSelect chip contains thousands of flow-isolated nanoliter chambers, called NanoPens, which physically isolate cells from flow but enable diffusion-based exchange. With the Beacon platform, tens of thousands of single cells can be isolated, cultured, assayed, and exported for further analysis across multiple OptoSelect chips.

BEACON® PLASMA CELL ANTIBODY DISCOVERY

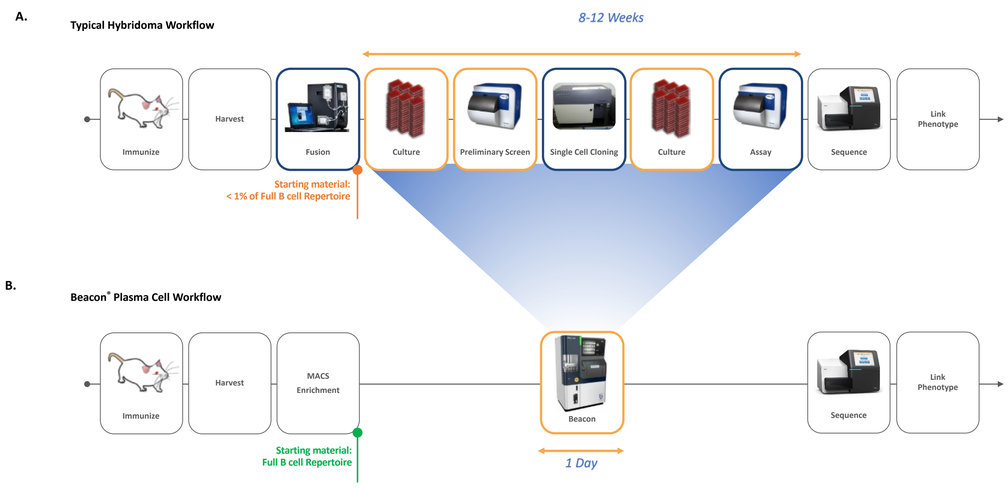


Figure 2.
(A) The typical hybridoma workflow starts with immunizations, harvest of tissue from immunized animals, and fusions. Fusions are plated out for primary and secondary screening of hybridoma supernatant. After one or more rounds of sub-cloning and repeating screening assays, antibodies are scaled up for purification and further characterization. The process post-fusion but prior to scale-up takes 8-12 weeks. (B) The Beacon platform condenses the 8-12 week workflow into one day by enabling direct evaluation of plasma B cells secreting target specific functional antibodies. Antibodies can be assayed for desired binding and blocking or other functional properties in tandem on the Beacon. Plasma B cells secreting antibodies with desired properties can be recovered by exporting, followed by cDNA amplification, VH/VL amplification and sequencing (e.g. Sanger or NGS). The process from harvesting and isolating B cells from immunized mice to VH/VL amplification can be done within three days or less, thus accelerating the antibody discovery process relative to the standard hybridoma workflow.

CHEMPARTNER ANTIBODY DISCOVERY WORKFLOW WITH B CELL CLONING

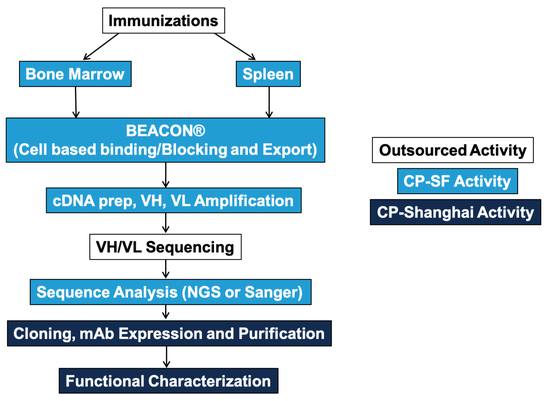


Figure 3. Proposed Plasma B Cell Cloning Workflow at ChemPartner
Immunizations were done at Pacific Biolabs using recombinant human PD-L1 ECD. After 10 weeks, bone marrow and spleen were harvested and isolations were done within 1-3 hours after harvest using the Miltenyi CD138 plasma B cell isolation kit. Isolated B cells were loaded onto Beacon and binding experiments were done in parallel using CHOK1-huPD-L1 cells and beads coated with huPD-L1. Subsequently, blocking experiments were done on both cells and beads using PD-1 AF488. Once positive binders and blockers were identified, single plasma B cells were exported into a 96 well plate for subsequent processing for single B cell sequencing. RNA was prepared from the lysed plasma B cells, followed by cDNA amplification, VH/VL amplification and sequencing (e.g. Sanger or NGS). For future workflows, B cell isolations through VH/VL amplification could be done at CP-SF. Sequencing will be done by an external vendor and, after data analysis, antibodies of interest will be cloned into expression vectors for expression, purification, and characterization at CP-Shanghai.

CD3, CD138, IgG ,AND IgM EXPRESSION ON CELLS PRE AND POST CD138 PLASMA B CELL ISOLATION

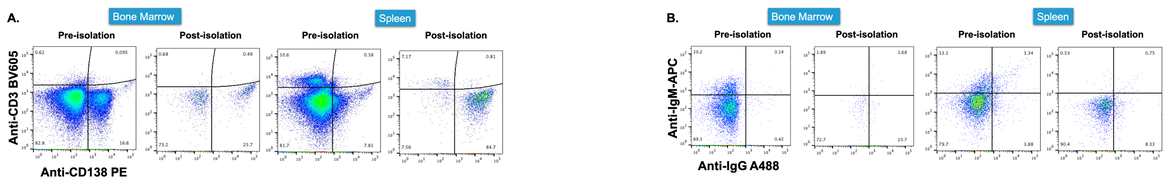


Figure 4. Flow Cytometry Analysis of Plasma B Cells
Cells were isolated from spleen and bone marrow with the Miltenyi CD138 isolation kit. Samples were taken pre and post-isolation and stained with anti-CD138 PE (Miltenyi, Clone REA104), anti-CD3 BV605 (Biolegend, Clone 17A2), anti-IgM Alexa Fluor 488 (Biolegend, Clone RMG1-1), anti-IgG1 APC (Thermo Fisher, Clone II/41) antibodies, and Zombie NIR (Biolegend, Live/Dead stain). (A) Analysis showed that a majority (~80%) of the cells from spleen were CD138+ with up to 10% CD3+ cells present, suggesting that T cells are a contaminating population within the isolated plasma B cell preparation from this organ. In contrast, the bone marrow plasma B cell preparation showed no CD3 staining with ~50% of the population being CD138+. (B) Cells were gated on CD138+ and CD3- staining prior to analysis of IgM and IgG staining profiles. Whereas IgM staining was evident prior to plasma B cell isolation, the CD138+ sub-population had minimal cells expressing IgM post-isolation in both the bone marrow and spleen preparations.

BINDING AND BLOCKING ASSAYS ON BEACON®

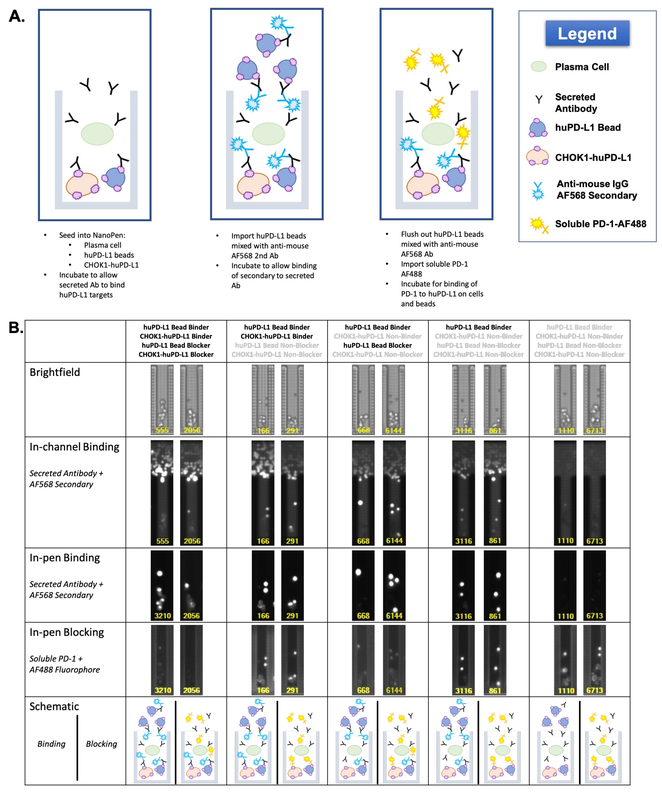
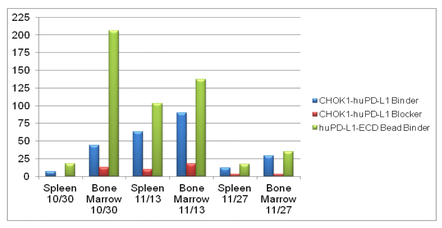


Figure 5. Representative Cell- and Bead-based Binding and Blocking on the Beacon® Platform
(A) For simultaneous cell- and bead-based binding assays, NanoPens were loaded with single plasma B cells, beads coated with huPD-L1 ECD, and CHOK1-huPD-L1 cells. Beads coated with huPD-L1 in solution with anti-mouse-IgG AF568 were also imported into the channels for a second huPD-L1 binding confirmation assay. If fluorescent signals were localized to cells or beads, this indicated target specific binding. After identifying cells secreting target specific binders, the beads coated with huPD-L1 in solution with anti-mouse IgG secondary antibodies were flushed out. For cell- and bead-based blocking assays, a soluble human PD-1 AF488 was then imported to determine if ligand could bind to cells or beads in the presence of target specific antibodies. (B) Representative images from the Beacon platform for in-channel/in-pen cell- and bead-based binding and blocking assays are shown. Target specific secreted antibodies are visualized with anti-mouse IgG AF568 on both cells and beads in-pen and in-channel for the binding assays. This enabled identification of NanoPens containing single plasma B cells secreting target specific binding and potentially blocking antibodies. Once the beads and secondary antibodies were washed away, human PD-1 AF488 was imported which enabled scoring of cell- and bead-based blocking signals as shown. If AF488 signals were weak in the presence of antibodies, these antibodies were considered to have ligand-blocking properties. If AF488 signals were strong along with strong cell- or bead-based binding, these were considered binders that did not have blocking activity.

BINDERS AND BLOCKERS IDENTIFIED ON BEACON®



	CHOK1-huPD-L1 Binder	CHOK1-huPD-L1 Blocker	huPD-L1-ECD Bead Binder	Single B cells on chip
Spleen 10/30	7	0	18	9535
Bone Marrow 10/30	44	13	206	3451
Spleen 11/13	63	10	103	4451
Bone Marrow 11/13	90	18	137	8285
Spleen 11/27	12	3	17	2514
Bone Marrow 11/27	29	3	35	5141
Total	**245**	**47**	**516**	**33377**

Figure 6. Binders and Blockers Identified on Beacon®
Results of three independent Beacon experiments are shown. Almost 13,000 single plasma B cells were evaluated in each of the first two experiments and over 7,600 single plasma B cells were analyzed in the third experiment. In the first experiment, significantly more cell- and bead-based binders were identified from the bone marrow compared to the spleen of immunized mice. In the second study, more binders were identified from both the bone marrow and spleen, with 18 ligand-blocking antibodies identified from the bone marrow and 10 from the spleen. In the third study, far fewer total binders were identified than the first two studies, however three blocking antibodies were identified each from the spleen and bone marrow. After evaluating over 33,000 single plasma B cells, a total of 47 plasma B cells secreting antibodies with cell based ligand-blocking properties were identified from the three studies combined. Of the 47 antibodies with blocking properties, 34 were isolated from bone marrow and 13 were isolated from spleen of immunized Balb/c mice.

HIERARCHICAL CLUSTERING TREE AT CDR3

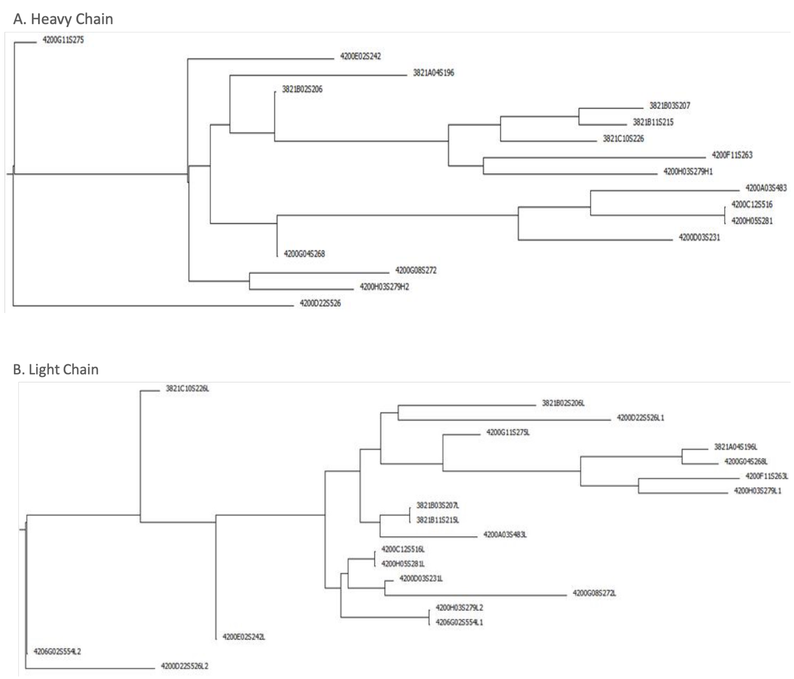


Figure 7. Hierarchical Clustering Tree of Heavy and Light Chain Sequences at CDR3 of Antibodies Identified on Beacon®
Single B cells from the study completed on 10/30/18 were exported from the Beacon platform and paired heavy chain and light chain (VH/VL) sequences were recovered using BLI's proprietary protocols. Sequencing was done using a MiSeq (Illumina, Inc., San Diego, CA) and paired VH/VL CDR3 sequences were recovered using BLI custom bioinformatics methods. Sequences were aligned to generate a hierarchical clustering tree for both heavy and light chain at CDR3. (A) Out of the CDR3 muti-sequence comparison results of 17 heavy chains, 16 were unique. (B) After alignment of the 20 light chain CDR3 sequences, 17 were identified as unique. Taken together, the results from the first study on the Beacon platform suggest greater sequence diversity at CDR3 of the heavy chain compared to that of the light chain.

CONCLUSIONS

The Beacon platform enabled accelerated identification of target specific binding and blocking antibodies in a fraction of the time that it takes to generate the same information from a standard hybridoma campaign. Binding activity was assessed on both cells and beads, followed by assessment of ligand-blocking activity on both. From two independent experiments, a total of 24 anti-huPD-L1 blocking antibodies were successfully exported and sequenced. The Beacon platform enables rapid discovery of target specific antibodies and interrogation of the immune repertoire across a variety of parameters, including:

- ✓Tissue type from immunized animals
- ✓Immunization strategy
- ✓Immunogen
- ✓Mouse strain

Taken together, the Beacon platform enabled rapid identification of anti-huPD-L1 functional antibodies to quickly ascertain which of the above parameters yields functional antibodies with desired properties. Importantly, by moving function forward, subsequent downstream activities, including sequencing, cloning, purification and characterization can be focused on clones with desired functional properties thus increasing the efficiency of the antibody discovery process.

REFERENCES

1. Caraux A. et al., Haematologica 2010 95(6):1016-1020
2. Swaika A. et al., Molecular Immunology 2015; 67(2A):4-17

ACKNOWLEDGEMENTS

The authors wish to thank Louis Liu and Sarah Lively for support and thoughtful discussions. We also thank Hui Wu and Yingying Hu for sequence analysis and generating hierarchical clustering trees.