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

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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 i.p. 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 taken at day 21 and 42 were measured by ELISA and titers increased between first and second boost. At day 57, spleen and bone marrow were harvested 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. Spleen cells were overlayed onto a Ficoll gradient to remove red blood cells. Cells from bone marrow or spleen were counted and kept separate throughout the isolation procedure using the CD138$^+$ mouse plasma cell isolation kit (Miltenyi Biotec). 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) 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 from single B cells using BLI's proprietary protocols. The protocols end with amplified whole mRNA, which can be further processed for targeted amplification or direct cloning workflows. Sanger sequencing was done on 22 clones to get variable region sequences from heavy and light chains corresponding to the blocking antibodies (ELIM Biopharmaceuticals, Inc., Hayward, CA).

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 three independent experiments, a total of 35 anti-huPD-L1 blocking antibodies were successfully exported and single VH and VL sequences were recovered from 22 clones. 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

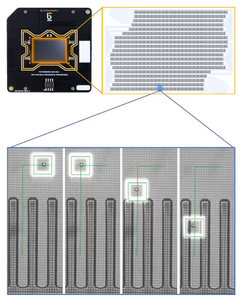
Taken together, the Beacon platform enabled rapid identification of anti-huPD-L1 functional antibodies to quickly ascertain which of the above parameters yields functional antibodies with desired properties. Importantly, by moving function forward, subsequent downstream activities, including sequencing,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

Introduction to BLI Technology

A. Beacon® Platform

B. OptoSelect™ Chip

Light manipulation + microfluidics



Single Cell Isolation in NanoPens™

Thousands in parallel

Figure 1. Introduction to BLI Technology

(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.

ChemPartner Antibody Discovery Workflow with B Cell Cloning

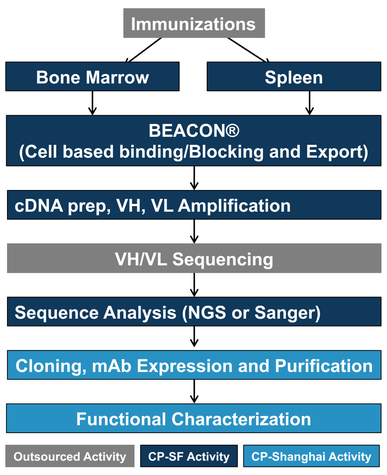


Figure 2. Proposed Plasma B cell Cloning Workflow at ChemPartner

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. 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. For future workflows, B cell isolations through VH/VL amplification could be done at CP-SF and antibodies of interest can be cloned, expressed, purified, and characterized at CP-Shanghai.

CD3, CD138, IgG, and IgM Expression on Cells Pre and Post CD138 Plasma B cell Isolation

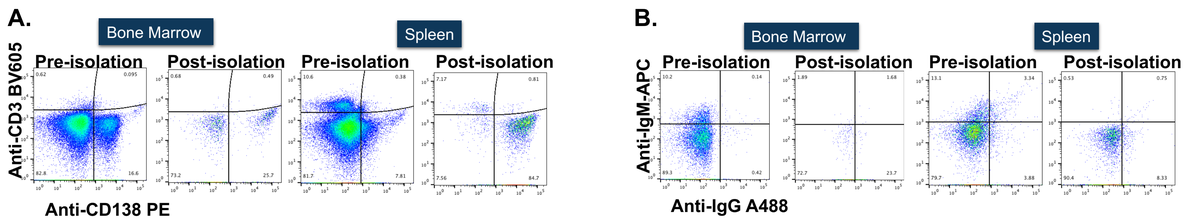
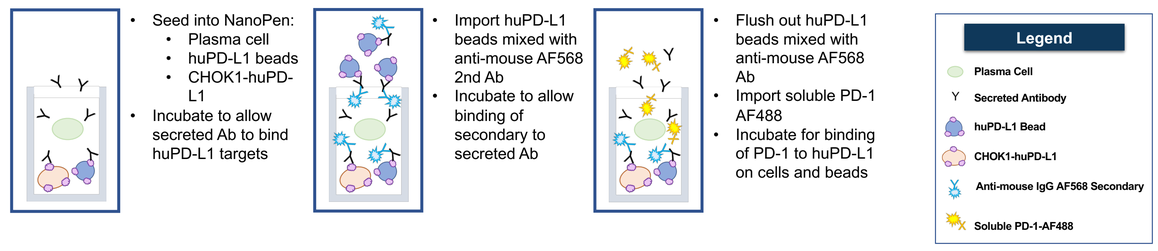


Figure 4. Flow Cytometry Analysis of Plasma B cells

After isolation of bone marrow and spleen cells using 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 cells from spleen were CD138+ with up to 10% CD3+ cells present, suggesting that T cells are a contaminating population in the isolated plasma B cells. 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 bone marrow and spleen preparations.

Binding and Blocking Assays on Beacon ®

A.



B.

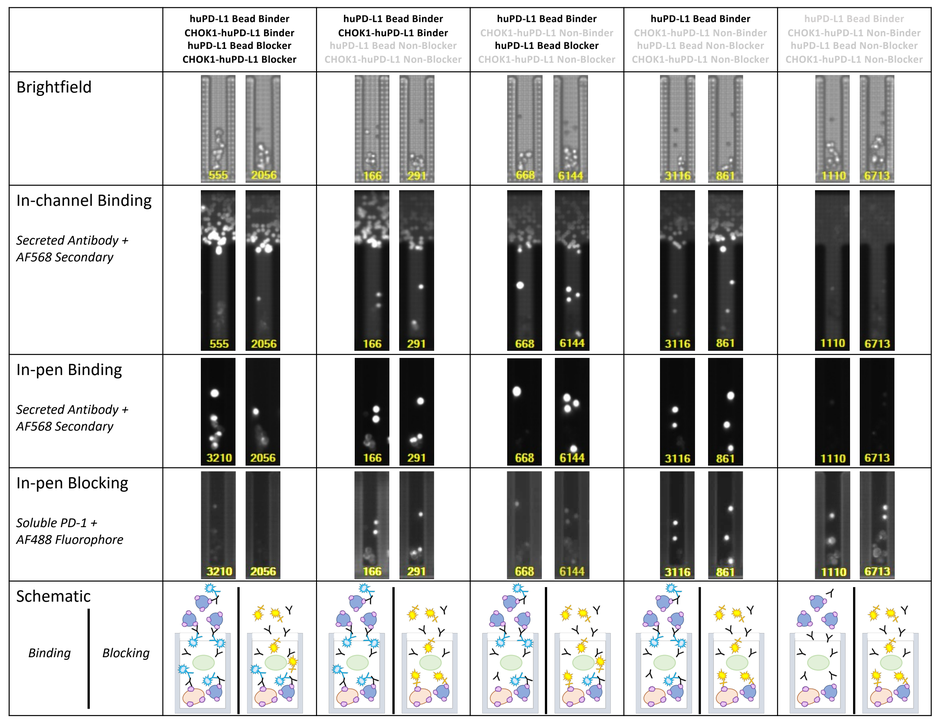
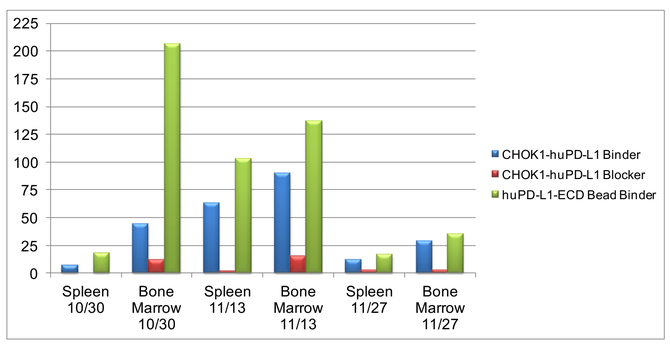


Figure 5. Representative Cell- and Bead-based Binding and Blocking on the Beacon® Platform

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 were flushed out. For cell- and bead-based blocking assays, a soluble human PD-1 AF488 was imported to determine if ligand could bind to cells or beads in presence of 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. Once beads and secondary antibodies were washed away, human PD-1 AF488 was imported to score cell- and bead-based blocking signals as shown.

Binding and Blocking Assays 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	12	206	3451
Spleen 11/13	63	2	103	4451
Bone Marrow 11/13	90	15	137	8285
Spleen 11/27	12	3	17	2514
Bone Marrow 11/27	29	3	35	5141
Total	**245**	**35**	**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 bone marrow compared to spleen of immunized mice. In the second study, more binders were identified from both the bone marrow and spleen, with 15 ligand-blocking antibodies identified from the bone marrow and 2 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 spleen and bone marrow. After evaluating over 33,000 single plasma B cells, a total of 35 plasma B cells secreting antibodies with cell based ligand-blocking properties were identified from three studies combined. Of the 35 antibodies with blocking properties, 30 were isolated from bone marrow and 5 were isolated from spleen of immunized Balb/c mice.

Hierarchical Clustering of Variable Region

A. Heavy Chain

B. Light Chain

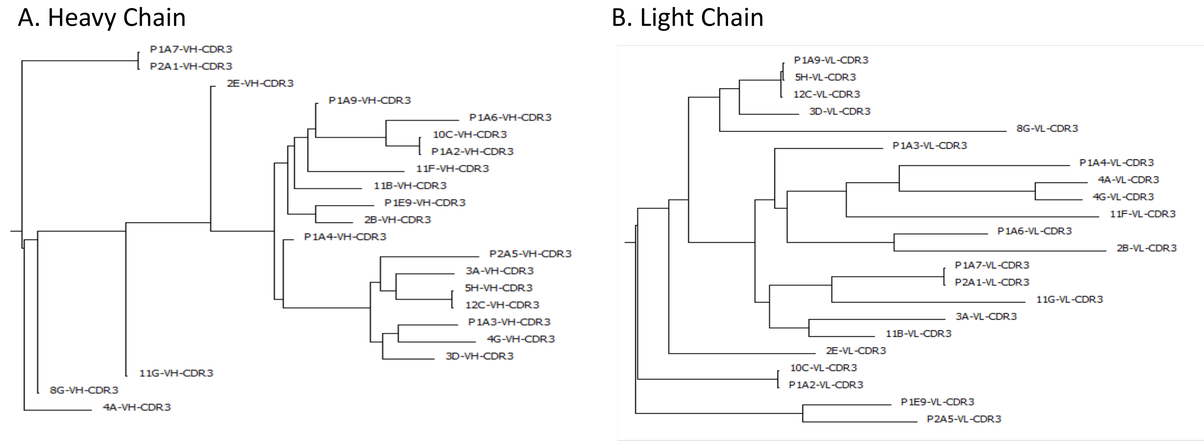


Figure 3. Hierarchical Clustering Tree of Heavy and Light Chain Sequences of Antibodies Identified on Beacon®

Single B cells from all studies were exported from the Beacon platform and paired heavy chain and light chain (VH/VL) sequences were recovered using BLI's proprietary protocols. Single VH and VL sequences were recovered from B cells secreting blocking antibodies and Sanger sequencing of the entire V region was completed on 22 clones (ELIM Biopharmaceuticals, Inc). V region sequences were aligned to generate a hierarchical clustering tree for both heavy and light chains. (A) Six sequences were identical corresponding to the V region of the heavy chain. (B) Seven of the light chain V region sequences were identical, however, due to pairing none of the 22 VH/VL sequences were identical. Taken together, the results on the Beacon platform suggest greater sequence diversity in the heavy chain V region compared to that of the light chain.

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

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