Functional Single Domain Antibodies Reactive to the Immune Checkpoint Targets PD-1 and PD-L1

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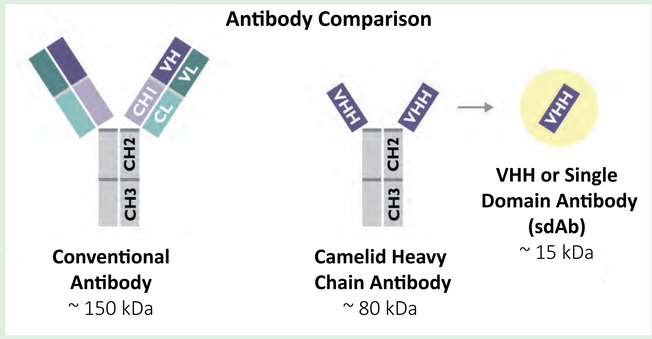
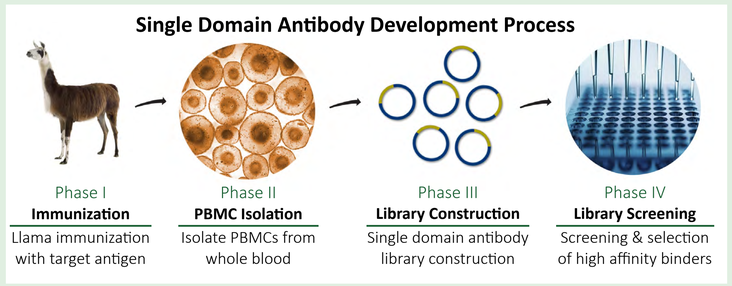
Abstract

Immune checkpoint antibodies are at the forefront of novel biologic treatments for cancers and others diseases. Here we present data on the development of both mouse monoclonal antibodies (mAbs) and camelid single domain antibodies (sdAbs) against the immune checkpoint regulators PD-1 and PD-L1. Their extracellular domains were expressed in HEK 293 cells and used for the immunizations of both mice and llamas. Mouse mAbs were developed by standard hybridoma technology while llama sdAbs were generated using phage display. These antibodies were characterized by ELISA, Western blot, immunocytochemistry (ICC), flow cytometry and epitope mapping. These mAbs and sdAbs were shown to bind to 293 cells that overexpress PD-1 or PD-L1 using flow cytometry and ICC. Two mouse PD-1 mAbs showed capture and detection capabilities in a sandwich ELISA format suggesting their pairing potential for diagnostic applications. The sdAbs against PD-1 and PD-L1 were also tested for functionality using a PD-1 blockage dependent T-cell activation reporter system, which demonstrated that at least one of each PD-1 and PD-L1 sdAbs has the ability to inhibit PD-1/PD-L1 interactions. To our knowledge, these are the first functional sdAbs developed against PD-1 and PD-L1. These results demonstrate that these immune checkpoint mAbs and sdAbs have research and diagnostic applications and may with further development have therapeutic potential.

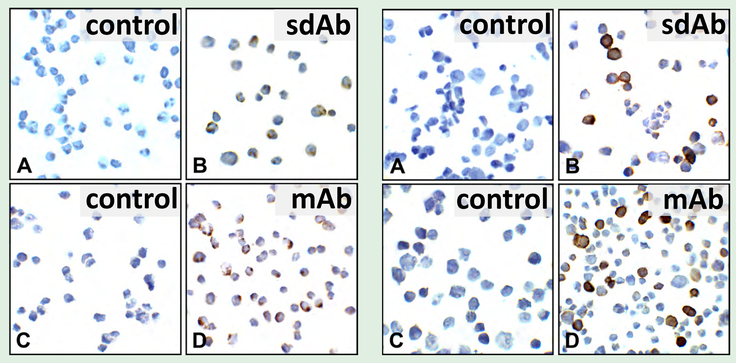
Development of Single Domain Antibodies





ProSci's Single Domain Antibody development process used to generate sdAbs against PD-1 and PD-L1.

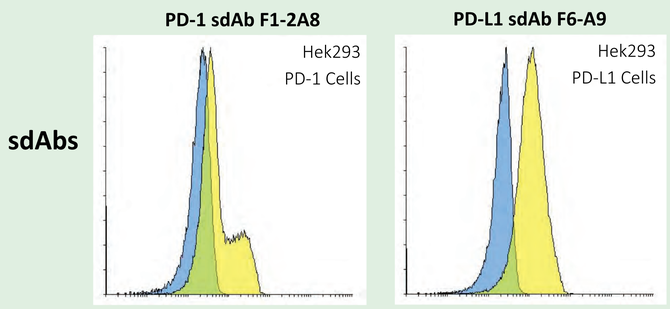
Immunocytochemistry Analysis of PD-1 and PD-L1 Antibodies



(A) Mouse anti-Myc-tag antibody followed by biotinylated anti-mouse antibody streptavidin-HRP (S/A-HRP). (B) Llama anti-PD-1 single domain antibody followed by mouse anti-Myc-tag antibody, then biotinylated anti-mouse antibody. (C) Mouse control IgG followed by biotinylated anti-mouse antibody. (D) Mouse monoclonal anti-PD-1 antibody followed by biotinylated anti-mouse antibody.

(A) Mouse anti-Myc-tag antibody followed by biotinylated anti-mouse antibody streptavidin-HRP (S/A-HRP). (B) Llama anti-PD-L1 single domain antibody followed by mouse anti-Myc-tag antibody, then biotinylated anti-mouse antibody. (C) Mouse control IgG followed by biotinylated anti-mouse antibody. (D) Mouse monoclonal anti-PD-L1 antibody followed by biotinylated anti-mouse antibody.

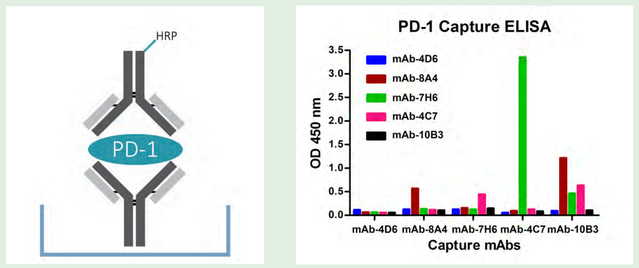
Flow Cytometry Analysis of PD-1 and PD-L1 Antibodies



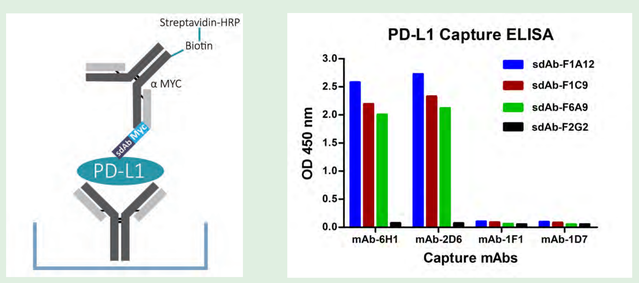
Single domain antibodies were analyzed for their ability to bind to PD-1 or PD-L1 expressed on the surface of transfected HEK293 cells.

Monoclonal antibodies directed against PD-1 and PD-L1 were analyzed for their ability to bind to their respective proteins expressed on the surface of HEK293 transfected cells.

PD-1 and PD-L1 ELISA Pairs

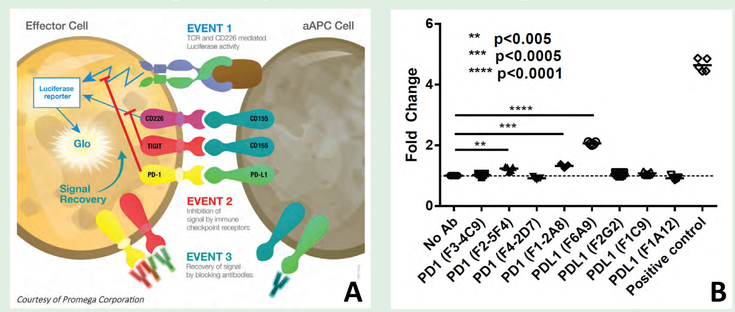


Capture mAb: Coat 500 ng of capture mAb in the well, incubate RT overnight.
PD-1 Protein: 50 ng of PD-1 protein in the well, incubate RT for 2 hours.
Detective mAb: Add 100 ng of each HRP labeled detective mAb in the well, incubate RT for 1 hour.



Capture mAb: Coat 1 ug of capture mAb in the well, incubate RT overnight
PD-L1 Protein: 50 ng of PD-L1 protein in the well, incubate RT for 2 hours
Detective single domain mAb: Add 1 ug of each single domain mAb in the well, incubate RT for 1 hour. Add 500 ng of anti-cMyc-HRP into each well, incubate RT for 1 hour.

Functional PD-1 and PD-L1 sdAbs



A) PD-1 engagement with PD-L1 inhibits T-cell receptor (TCR) signaling and subsequent TCR-mediated proliferation, activation and cytokine production. The PD-1/PD-L1 Blockade Bioassay is a biologically relevant MOA-based assay used to measure the potency and stability of antibodies and other biologics designed to block the PD-1/PD-L1 interaction. The assay consists of two genetically engineered cell lines:

1) PD-1 Effector Cells: Jurkat T cells stably expressing human PD-1 and NFAT-induced luciferase.
2) PD-L1 aAPC/CHO-K1 Cells: CHO-K1 cells stably expressing human PD-L1 and a cell surface protein designed to activate cognate TCRs in an antigen-independent manner.

B) PD-L1 APCs were added to the 96 well plate. 18 hours later PD-1 or PD-L1 antibodies along with PD-1 effector cells were added. 6 hours later Bio-Glo™ luciferase substrate added and the absorbance signal read.

Summary of Mouse mAbs and Llama sdAbs Developed Against PD-1 and PD-L1

Mouse mAbs

Product	Cat. No.	Clone Name	ELISA	WB	ICC	IF	IHC	Flow
PD-1	RF-16001	4D6	++++	++++	+++	+++	+++	-
PD-1	RF-16002	8A4	++++	++++	++	++	+++	-
PD-1	RF-16003	7H6	++++	++	++	++	+++	++
PD-1	RF-16004	4C7	++++	-	++	+	++	+
PD-1	RF-16005	10B3	++++	-	+	+	++	+
PD-L1	RF-16031	4F2	++++	++++	++++	ND	ND	++++
PD-L1	RF-16032	8E12	++++	++++	++++	ND	ND	++++

Llama Single Domain mAbs

Product	Cat. No.	Clone Name	ELISA	WB	ICC	Flow	Functionality
PD-1	PM-8643	F1-2A8	++++	-	-	+	++
PD-1	PM-8645	F4-2D7	++++	-	-	+	-
PD-1	PM-8647	F3-4C9	++++	-	++	+	-
PD-1	PM-8649	F2-5F4	++++	-	-	+	+
PD-L1	PM-8585	F1A12	++++	-	+++	++	-
PD-L1	PM-8623	F1C9	++++	-	+++	++	-
PD-L1	PM-8639	F6A9	++++	-	-	++	+++
PD-L1	PM-8641	F2G2	++++	-	-	-	-

Western blots, immunocytochemistry staining, immunofluorescence and flow cytometry analysis were performed using overexpressing PD-1 or PD-L1 293 cells and cell lysates.

Summary

PD-1 is an immune inhibitory receptor crucial for immune regulation found on activated T and B cells. PD-L1 and PD-L2 ligands on tumor cells can inhibit T-cell receptor (TCR) signaling and activation upon engagement with the PD-1 receptor on T-cells which can allow tumor cells to evade targeting. Antibodies against PD-1 and PD-L1/PD-L2 that block this TCR signaling inhibition are proving useful for the treatment of a variety of cancers. Both mouse monoclonal antibodies (mAbs) and camelid single domain antibodies (sdAbs) against PD-1 and PD-L1 were developed and characterized. ELISA pairs and antibodies useful for Western blot, immunocytochemistry and flow cytometry were generated. Several sdAbs against PD-1 and PD-L1 were further tested for functionality using a PD-1/PD-L1 blockage bioassay. sdAbs against PD-1 and PD-L1 both demonstrated functionality by blocking the PD-1/PD-L1 signaling pathways and preventing TCR inhibition. To our knowledge, these are the first functional sdAbs developed against PD-1 and PD-L1. These mAbs and sdAbs developed against PD-1 and PD-L1 can be useful tools for research and diagnostics and may have therapeutic potential.