Isolation and characterization of a promising anti-PD-1 antibody to treat cancer

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ABSTRACT

Antibodies targeting checkpoint inhibitors have been shown to be a very effective therapy to treat cancer, being PD-1 (Programmed cell death protein 1) one of most successful targets to develop antibody-based drugs. PD-1 is expressed on the surface of T and B lymphocytes and transmits inhibitory signals when binds to its ligands PD-L1 and PD-L2 expressed on macrophages and dendritic cells. PD-L1/L2 are also highly expressed in several cancer cells resulting in immune evasion of tumor cells. Hence, blockade of the PD-1:PD-L1/L2 interaction by monoclonal antibodies unleashes the immune system to destroy cancer cells.

In this work we used a fully synthetic phage display library to generate a panel of anti-PD-1 antibodies with diverse binding and functional profiles. After three rounds of panning in solution with recombinant PD-1 as a selector, 315 clones were tested for PD-1 binding. Out of these clones, 143 were specific for PD-1, with 60 being unique. Further characterization of this panel of PD-1 binders and conversion of the best performing clones to IgG4PE, resulted in an antibody called D9 that blocked PD-1:PD-L1/L2 interaction in ELISA and Junkat cells, and was not cross-reactive with CD28 family-related molecules. C9 also promoted expression of Interferon gamma in a mixed lymphocyte reaction co-culture assay comparable to FDA-approved anti-PD1 therapeutic antibodies Keytruda and Obdivo. Therefore, C9 seems to be a good lead candidate for development of an antibody-based drug targeting checkpoint inhibitors as a therapy to treat cancer.

METHODOLOGY

1.Phage display, panning, screening and selection of candidates.

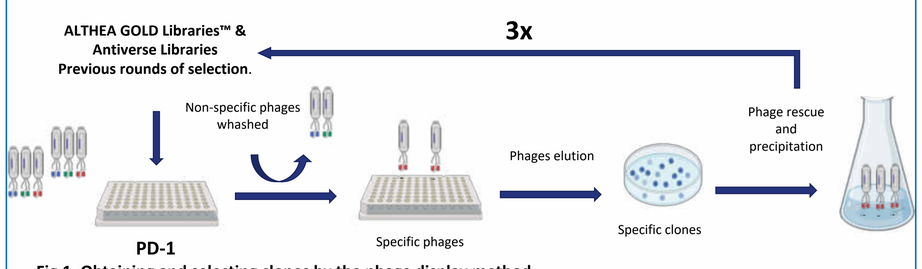


Fig.1. Obtaining and selecting clones by the phage display method

2. Obtaining clones in IgG4 format.

Transfection of EXPI293F cells and conversion to IgG4 and purification by HPLC in supernatant (6 clones).

3.Antibodies anti-PD-1 biological characterization.

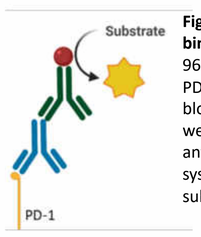


Fig.2. Anti-PD-1 antibodies binding to human PD-1 by ELISA. 96 wells plate were coated with PD1, incubated over night and blocked, anti-PD1 antibodies were added. As detection agent anti-IgG-HRP was used . The system was revealed with TMB substrate Reagent.

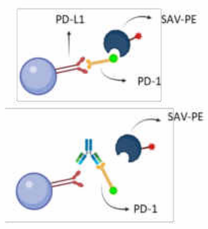


Fig.3. Blocking of PD1:PDL-1 interaction by Anti-PD-1 antibodies . PDL-1 was coupled to SAV beads, and a mix of anti-PD1 antibodies and PD1-AVI was added . the interaction was revealed with streptavidin coupled to phycoerythrin and MIF.

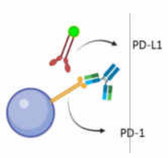


Fig.4. Competition assay for the PD-1 receptor by flow cytometry PD-1 was coupled SAV beads, then a 1:1 mixture of anti-PD1 antibody and PD-L1 ligand was added at constant concentration. The assay was revealed with streptavidin-PE.

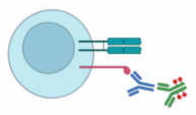


Fig.5. Binding to human PD-1 in cells. Cells were recovered and treated with anti-PD1 antibodies, subsequently binding was revealed with a secondary antibody coupled to phycoerythrin.

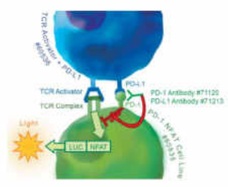


Fig.6. Blocking in nfat reporter jurkat cells. The first of these is the CHO-K1 line that constitutively expresses human PD-L1 and a T cell receptor (TCR) activator. The second of these is the recombinant Jurkat line constitutively expressing human PD-1 and with conditional expression of the firefly luciferase gene under the control of NFAT response elements located upstream of the TATA promoter. Therefore, NFAT stimulation can be monitored by measuring luciferase activity, which was detected in the Spectramax equipment by means of emitted luminescence.

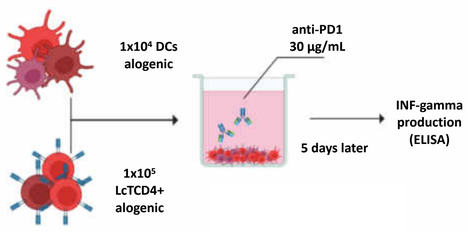


Fig.7. Mixed lymphocyte reaction Dendritic cells (DC) will be obtained by culturing monocytes purified from PBMC's using a MACS miltenyi in vitro magnetic bead purification kit and cultured by adding stimuli for 7 days. The added stimuli will be 2.5 µg/mL of interleukin-4 (IL-4) and 2.5 µg/mL of GM-CSF. CD4+ T cells ($1x10^5$) and allogeneic DC ($1x10^4$) were co-cultured with or without different concentrations of anti-PD-1 antibodies added at baseline. After 4 days, the IFN-γ secretion in the culture supernatants was evaluated by ELISA.

RESULTS

1.Phage display, panning, screening and selection of candidates

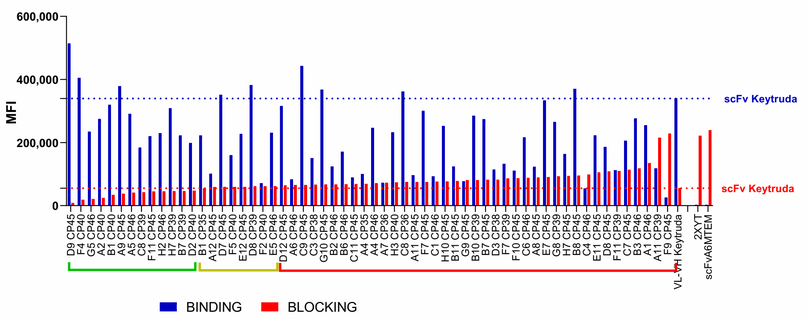


Fig.8. Selection of clones obtained from phage display. The green bar indicates the 13 clones that have the best ability to block the binding of the PD-L1 ligand with the human PD-1 receptor, the yellow bar shows the clones that have the least blocking ability, and the bar red are those with the least ability to block.

2. Obtaining clones in IgG4 format.

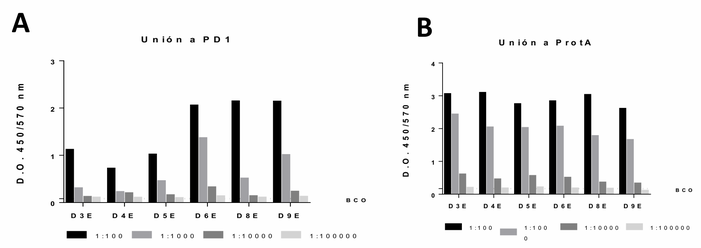
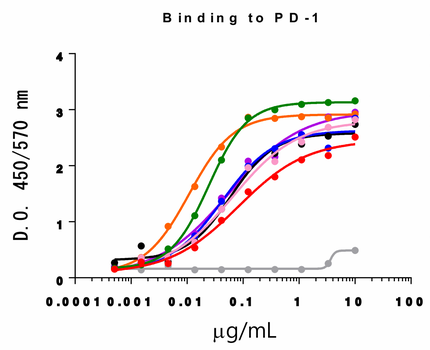


Fig.9. Evaluation of transfection supernatants. Of the 13 clones, the 6 clones that had the best blocking capacity were selected and the conversion to IgG4 was carried out by transfecting into EXPI293F cells.A) The determination of specific IgGs to PD-1 in the transfection supernatants is shown. A and B) Binding to PD-1 of clones D3E, D4E, D5E, D6E, D8E and D9E by ELISA

3.Antibodies anti-PD-1 biological characterization.



Expression system	
EXPI293F	
scFv	IgG
B1 CP40	D3E
F4 CP40	D4E
A9 CP45	D5E
D9 CP45	D6E
A5 CP46	D8E
H2 CP46	D9E

Fig.10. Binding to human PD-1 of purified antibodies. The 6 clones, when converted to IgG4 format, had their names changed (See table).An ELISA binding to human PD-1 was performed by placing 1 µg/mL on the plate, antibodies were evaluated starting at 10 µg/mL..

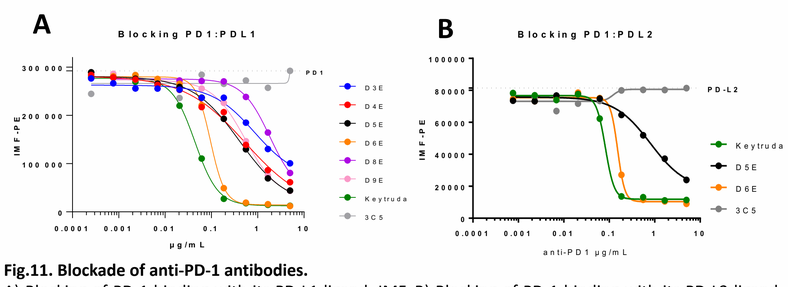


Fig.11. Blockade of anti-PD-1 antibodies. A) Blocking of PD-1 binding with its PD-L1 ligand, IMF. B) Blocking of PD-1 binding with its PD-L2 ligand, IMF

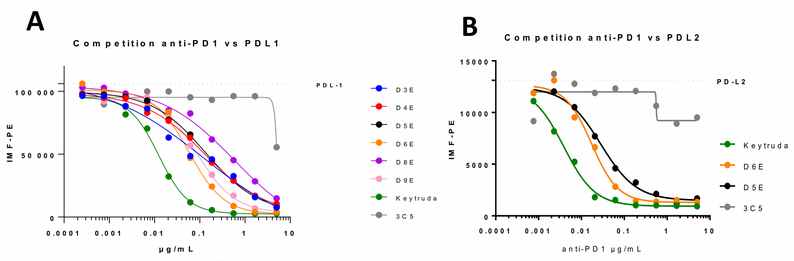


Fig.12. Competition of anti-PD-1 antibodies. A) Competition for the binding of PD-1 with its ligand PD-L1, IMF. B) Competition for the binding of PD-1 with its ligand PD-L2, IMF

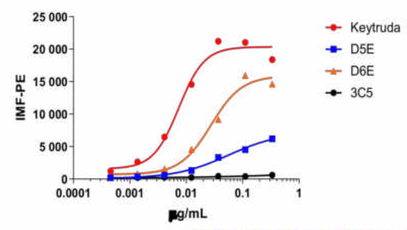


Fig.13. Binding to native PD-1 in Jurkat cells of clones D6E and D5E Binding was determined by flow cytometry using an anti-human Fc IgG antibody coupled to phycoerythrin (PE), 10,000 events were captured for each sample.

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RESULTS

Fig.14. In vitro PD-1:PDL-1 binding blocking assay. Co-cultivation of Jurkat cells with CHOK1 cells in vitro in blocking assay to mediate anti-PD1 antibody activity.

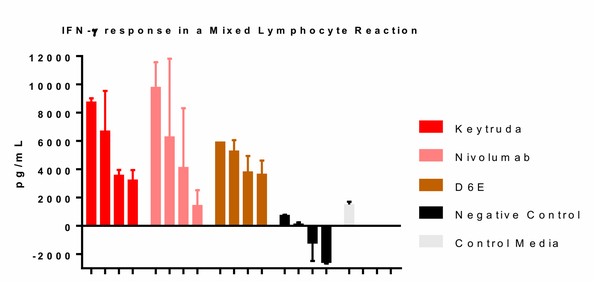

Fig.15.IFN γ production in supernatant. The supernatants were recovered and the final concentration of IFN γ in each evaluated condition was calculated. It is observed that D6 has the ability to induce IFN γ production.

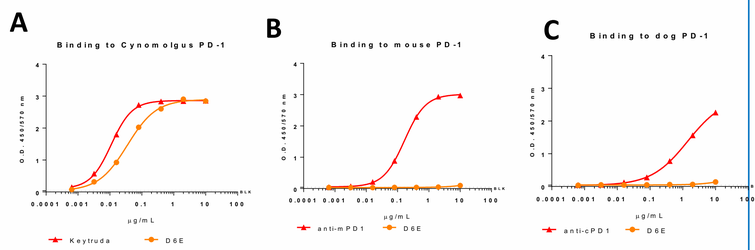

Fig.16. ELISA binding to mouse, canine and monkey (Cynomolgus) PD-1. A) ELISA DE unión a PD-1 de mono (Cynomolgus). B) ELISA de unión a PD-1 de ratón, C) Unión a PD-1 canino.

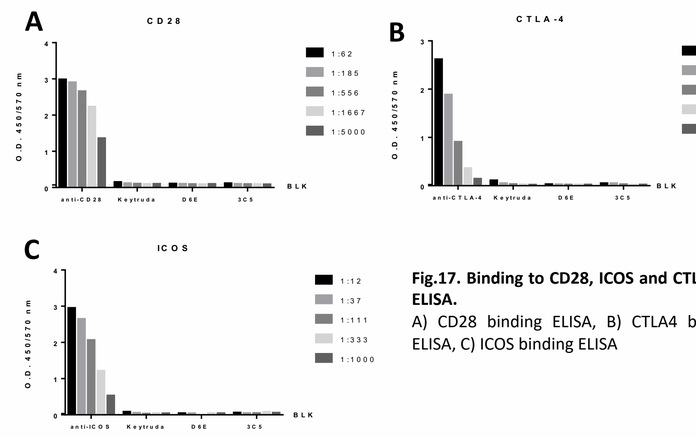

Fig.17. Binding to CD28, ICOS and CTLA4 by ELISA. A) CD28 binding ELISA, B) CTLA4 binding ELISA, C) ICOS binding ELISA

CONCLUSIONS

A robust functional characterization of antibodies from early stages of their development supports their quality and functionality. Within the characteristics to be evaluated in the discovery stage, the biological characterization is fundamental including recognition of its target, selectivity and blocking of the interaction with relevant receptors.

The D6 antibody has the ability to block binding to PD-L1 and PD-L2 ligands. It also did not show evidence of having affinity with related molecules of the same PD-1 family such as ICOS, CD28 and CTLA-4. In addition, it was possible to observe the effect on the production of IFN γ in a co-culture of CD4+ T lymphocytes with allogeneic dendritic cells, this being what is expected for an antibody that has the capacity to block the union with its natural ligands, making it a promising candidate to develop for immunotherapy.

REFERENCES

1. *Keir, M. E., Butte, M. J., Freeman, G. J., & Sharpe, A. H. (2008). PD-1 and its ligands in tolerance and immunity. Annu. Rev. Immunol., 26, 677-704.*
2. *Chen, L., & Han, X. (2015). Anti–PD-1/PD-L1 therapy of human cancer: past, present, and future. The Journal of clinical investigation, 125(9), 3384-3391.*
3. *Zak, K. M., Grudnik, P., Magiera, K., Dömling, A., Dubin, G., & Holak, T. A. (2017). Structural biology of the immune checkpoint receptor PD-1 and its ligands PD-L1/PD-L2. Structure, 25(8), 1163-1174.*
4. *Han, Y., Liu, D., & Li, L. (2020). PD-1/PD-L1 pathway: current researches in cancer. American journal of cancer research, 10(3), 727.*
5. *David Goodsell, December 2016, doi:10.2210/rcsb_pdb/mom_2016_12*

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