Blockade of Programmed Death-Ligand 1 with Atezolizumab in Human Breast Cancer 3D Spheroid Colonies Induces Changes in Cell Viability

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Blockade of Programmed Death-Ligand 1 with Atezolizumab in Human Breast Cancer 3D Spheroid Colonies Induces Changes in Cell Viability

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Introduction

- Solid tumours are characterised by a three-dimensional (3D) architecture that provides specific survival advantages such as resistance to anti-cancer drugs (Rios de la Rosa *et al.,* 2018).
- The expression of programmed death-ligand 1 (PD-L1) is one such survival mechanism employed by tumours to mediate immune evasion, drug resistance and tumour progression (Dong *et al.,* 2018).
- PD-L1 overexpression by cancer cells and immune cells in the tumour microenvironment is well known to promote immune evasion, primarily by inhibition of cytotoxic T lymphocyte and natural killer (NK) cell effector function (Jiang *et al.,* 2019). However, tumour-intrinsic PD-L1 signalling is less established.
- Monoclonal antibodies targeting PD-L1 have demonstrated significant anti-tumour immune responses in select patients with advanced cancers (Yang and Hu, 2019)
- Better mimicking of the 3D architecture of solid tumours by utilising 3D cell culture could provide an environment more representative of *in vivo* human tumours for the investigation of PD-L1 in relation to its tumour-intrinsic role, interaction with PD-1 on immune cells and response to treatment.

Aims and Hypothesis

- This research aimed to: **1)** determine whether PD-L1 expression by human breast cancer cells altered in 3D cell culture compared to 2D cell culture and **2)** investigate the effects of PD-L1 blockade ± cytokine modulation on human breast cancer cell viability in a 3D cell culture system.
- It was hypothesised that PD-L1 expression would alter in 3D cell culture compared to 2D cell culture and that PD-L1 blockade ± cytokine modulation would promote cancer cell death in human breast cancer cells expressing moderate to high levels of PD-L1 following growth in a 3D cell culture system.

Methods

Cell Culture Human breast cancer cells (MDA-MB-231 and MCF-7) were cultured in RPMI media supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were cultured in 2D monolayer and 3D alginate hydrogel beads (Figure 1). Cells were purchased from ATCC, frequently tested for mycoplasma and were below passage 20 for all experiments.

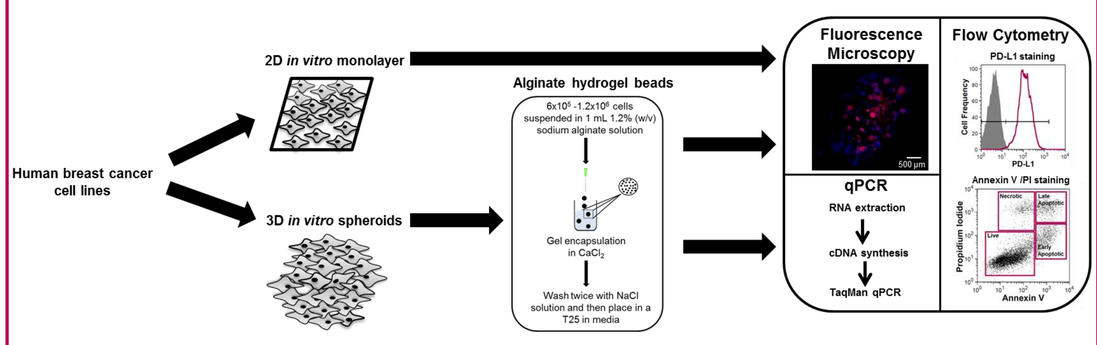


Figure 1. A schematic diagram of the work flow applied to human breast cancer cells to generate 2D and 3D cultures for assessment using fluorescent microscopy, qPCR and flow cytometry. For 3D cell culture, alginate hydrogel beads were utilised which facilitate the formation of spheroid colonies that display heterogeneous population of cells resembling that found in the tumour microenvironment. Spheroid colonies generated were harvested for downstream analysis at day 3, 6 and 10. 2D-cultured cells were harvested 3 days after seeding at day 3, 6 and 10. Appropriate controls and gating strategies were carried out for each experiment.

Cell Viability in 3D Culture At day 3, 6 and 10 alginate hydrogel beads for each cell line were harvested from culture, placed in a 96-well plate and labelled with Hoechst 33342 (10 µg/mL) and propidium iodide (PI) (10 µg/mL). Images were captured using cellSense Software on an Olympus IX81 microscope.

PD-L1 Expression Total RNA from 2D- and 3D- cultured breast cancer cells was extracted, quantified and converted to cDNA for measuring mRNA levels of PD-L1 using TaqMan qPCR. Cell surface PD-L1 expression was assessed using flow cytometry by staining with fluorescently labelled anti-human PD-L1 and matched isotype control. Data was acquired using a BD FACSCalibur or Beckman Coulter CytoFLEX and analysed using FlowJo software. The median fluorescent intensity (MFI) was normalised relative to the isotype control.

Atezolizumab Treatment Cells grown in 3D cell culture were treated with 10 nM Atezolizumab ± IFNγ and/or TNFα over a time course of 10 days (Figure 2 A and B). Cells were harvested and assessed by flow cytometry for cell surface PD-L1 expression. Simultaneously, cells were stained with Annexin V and PI to assess cancer cell death using flow cytometry. Some cells were treated with IFNγ (0.5 ng/ml) and TNFα (5 ng/ml) for 48 hr before Atezolizumab treatment to modulate PD-L1 expression.

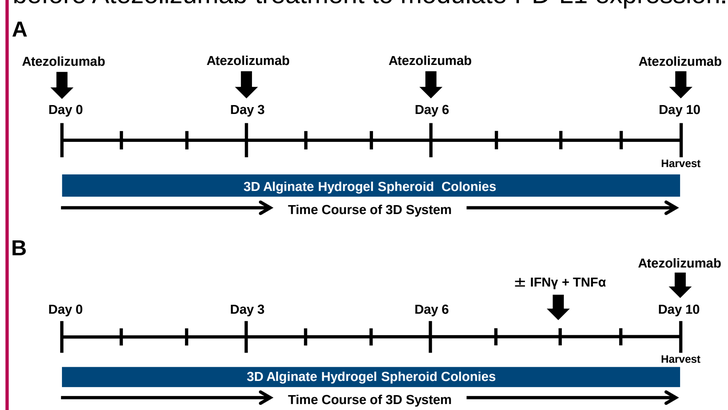


Figure 2. Timeline of Atezolizumab treatment strategies ± IFNγ and/or TNFα for 3D-cultured breast cancer cells. (A) 3D cultures were treated with Atezolizumab every 3-4 days from day 0. Following this treatment strategy, 3D cultures were harvested for analysis at day 10. **(B)** 3D cultures were treated with IFNγ and/or TNFα 48 hr prior to dosing with Atezolizumab at day 10 for 1 hr before harvesting for analysis. Appropriate controls were utilised in each experiment.

Results

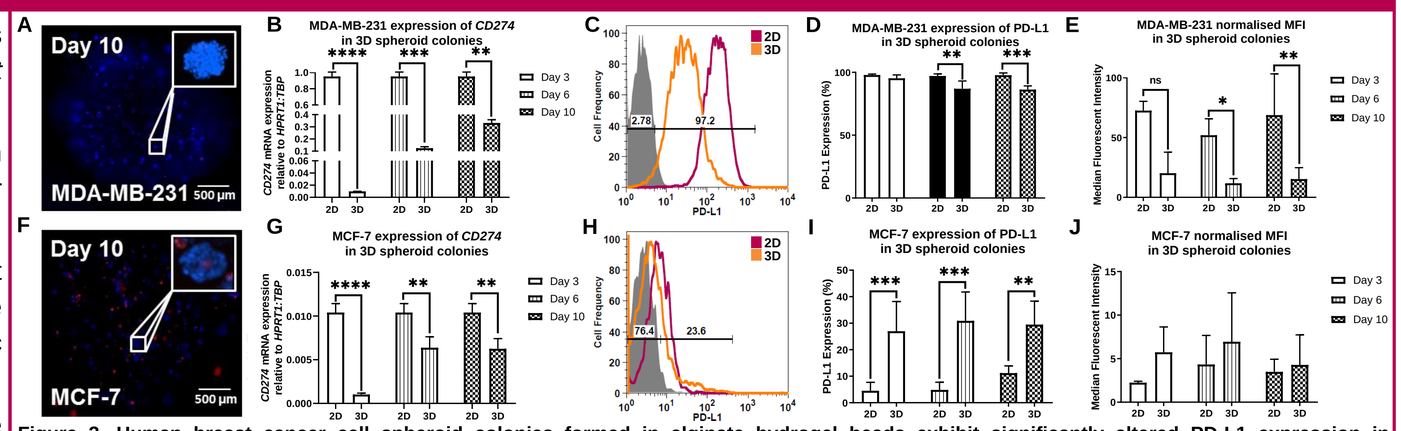


Figure 3. Human breast cancer cell spheroid colonies formed in alginate hydrogel beads exhibit significantly altered PD-L1 expression in comparison to their 2D counterparts at mRNA and protein level. Fluorescent images obtained by Hoechst 33342/PI staining of whole intact alginate beads at day 10 are shown for each breast cancer cell line; MDA-MB-231 **(A)** and MCF-7 **(F)**. Scale bar represents 500 µm. The closer images of individual spheroid colonies in the white boxes represent spheroids within the alginate bead that have a diameter of 100-200 µm for each cell line. MDA-MB-231 cancer cells grown in 3D display a significant decrease in the level of PD-L1 (*CD274*) mRNA **(B)** and protein (**C, D, E**) at day 3, 6 and 10 compared to when grown in 2D. Similarly, MCF-7 cancer cells in 3D exhibit a significant decrease in PD-L1 (*CD274*) mRNA at day 3, 6 and 10 **(G)**. Interestingly, MCF-7 cancer cells show a significant increase in the frequency of cells expressing PD-L1 expression at each time point investigated in 3D in comparison to their 2D counterparts **(H, I)**, but display no change in the level of PD-L1 at the protein level **(J)**. Data is presented as mean ± SD, n=3 independent experiments each with 3 technical repeats, Kruskal-Wallis followed by Conover Inman multiple comparison test, *P<0.05, **0.01, ***0.001, ****P<0.0001.

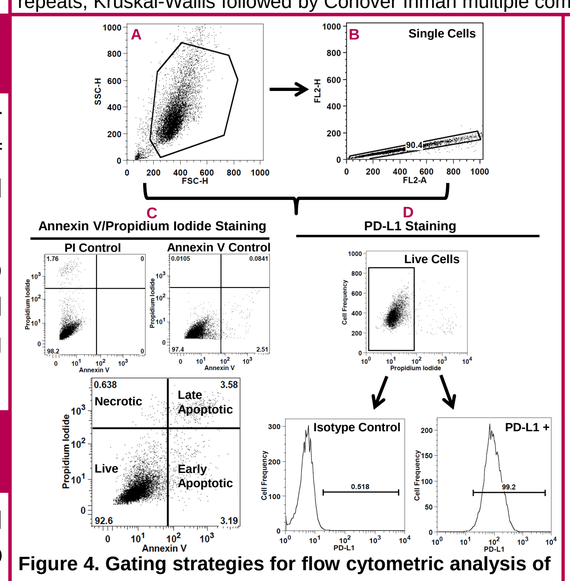


Figure 4. Gating strategies for flow cytometric analysis of cell surface PD-L1 and cell death via Annexin V/PI staining. For all experiments cells of interest **(A)** and single cell populations **(B)** were gated. Single colour controls for Annexin V/PI staining were used to gate for live (AV-/PI-), early apoptotic (AV+/PI-), late apoptotic (AV+/PI+) and necrotic (AV-/PI+) cells **(C)**. Live cell gating was performed for assessing PD-L1 expression by staining with PI. Isotype controls for untreated and treated cells were prepared and used to gate for PD-L1 negative populations **(D)**.

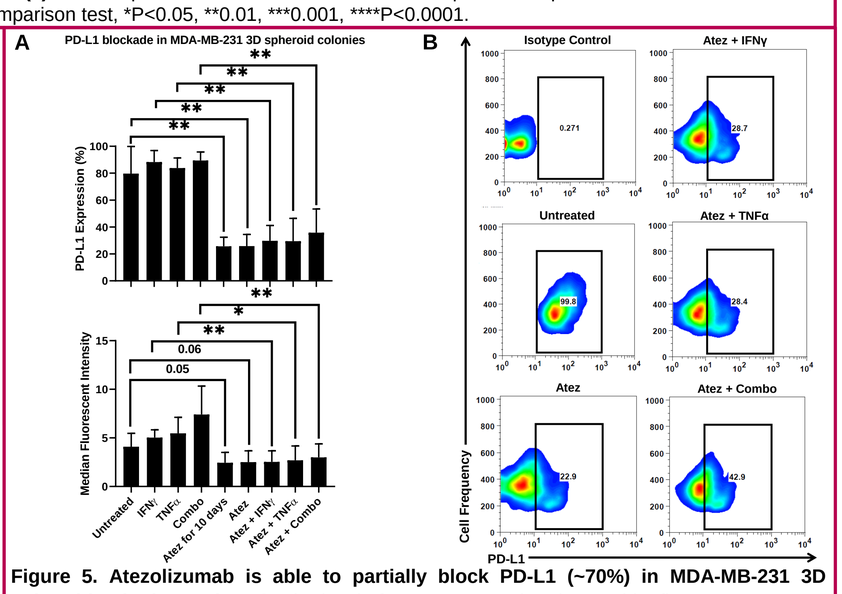
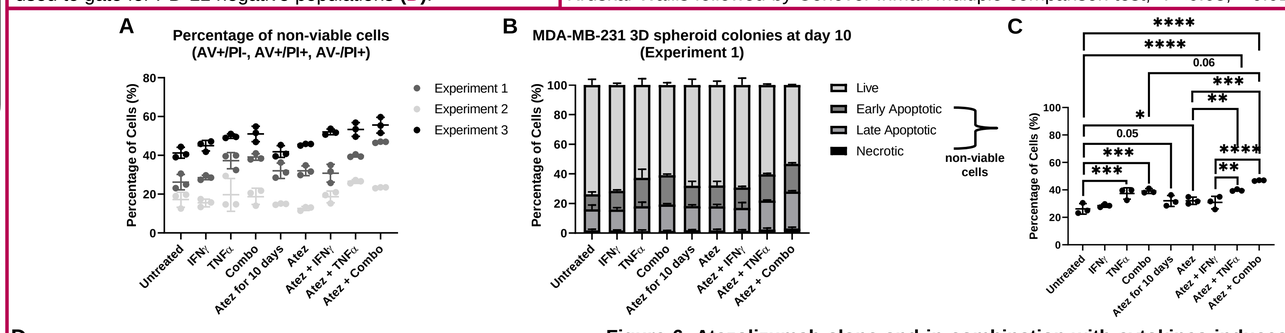


Figure 5. Atezolizumab is able to partially block PD-L1 (~70%) in MDA-MB-231 3D spheroid colonies at day 10. The level of PD-L1 expression detected by flow cytometry was reduced by ~70% when blocked with Atezolizumab in 3D spheroid colonies at day 10 **(A).** Whether Atezolizumab alone or combined with cytokines, detectable PD-L1 expression was significantly reduced compared to untreated and cytokine only treated cells. Representative flow cytometry plots illustrate the degree of blockade of PD-L1 by Atezolizumab in 3D spheroid colonies treated with Atezolizumab alone or combined with cytokines **(B)**. There is a trend showing that less PD-L1 is blocked when Atezolizumab is combined with both cytokines. Data is presented as mean ± SD, n=3 independent experiments each with 3 technical repeats, Kruskal-Wallis followed by Conover Inman multiple comparison test, *P<0.05, **0.01.



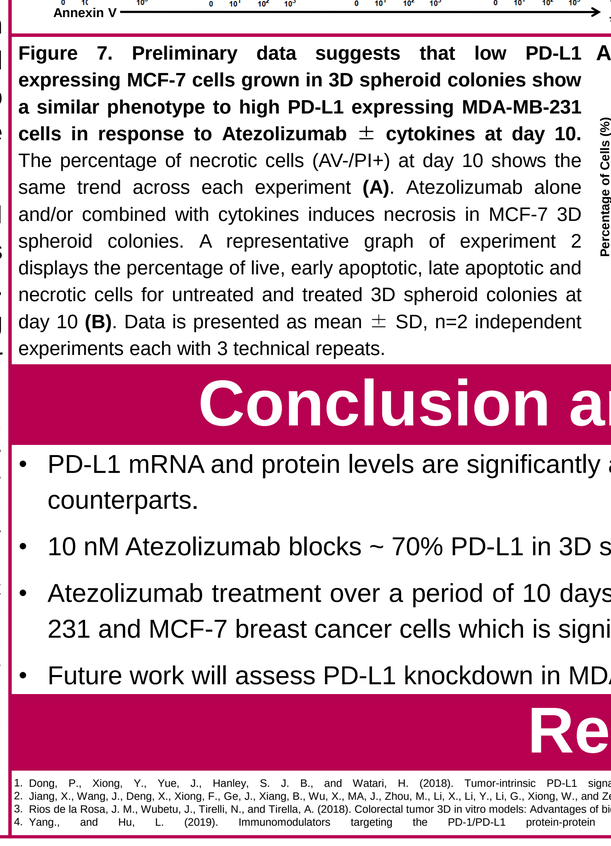


Figure 6. Atezolizumab alone and in combination with cytokines induces cell death in MDA-MB-231 3D spheroid colonies at day 10. The percentage of non-viable cells (AV+/PI-, AV+/PI+, AV-/PI+) at day 10 shows the same trend across each experiment **(A)**. Atezolizumab alone and/or combined with cytokines induces cell death in MDA-MB-231 3D spheroid colonies at day 10. A representative graph of experiment 1 demonstrates the percentage of live, early apoptotic, late apoptotic and necrotic cells for untreated and treated 3D spheroid colonies at day 10 **(B)**. Atezolizumab alone and cytokines alone induce cell death in 3D spheroid colonies at day 10 and when Atezolizumab is combined with cytokines there is a synergistic effect inducing cell death in 3D spheroid colonies; which is significant when Atezolizumab is combined with both cytokines **(C, D)**. Data is presented as mean ± SD, n=3 independent experiments each with 3 technical repeats, Kruskal-Wallis followed by Conover Inman multiple comparison test, *P<0.05, **0.01, ***0.001, ****P<0.0001.

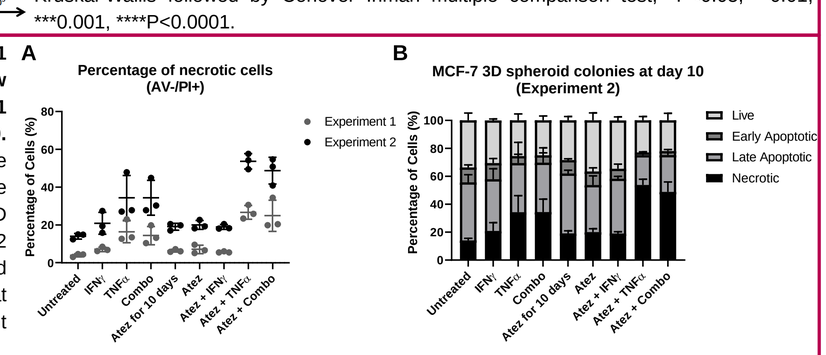


Figure 7. Preliminary data suggests that low PD-L1 expressing MCF-7 cells grown in 3D spheroid colonies show a similar phenotype to high PD-L1 expressing MDA-MB-231 cells in response to Atezolizumab ± cytokines at day 10. The percentage of necrotic cells (AV-/PI+) at day 10 shows the same trend across each experiment **(A)**. Atezolizumab alone and/or combined with cytokines induces necrosis in MCF-7 3D spheroid colonies. A representative graph of experiment 2 displays the percentage of live, early apoptotic, late apoptotic and necrotic cells for untreated and treated 3D spheroid colonies at day 10 **(B)**. Data is presented as mean ± SD, n=2 independent experiments each with 3 technical repeats.

Conclusion and Future Direction

- PD-L1 mRNA and protein levels are significantly altered in breast cancer cells in 3D cell culture compared to their 2D counterparts.
- 10 nM Atezolizumab blocks ~ 70% PD-L1 in 3D spheroid colonies .
- Atezolizumab treatment over a period of 10 days in 3D spheroid colonies induces increased cell death of MDA-MB-231 and MCF-7 breast cancer cells which is significantly enhanced via cytokine modulation.
- Future work will assess PD-L1 knockdown in MDA-MB-231 breast cancer cells cultured in 2D and 3D cell culture.

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