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Programmed death-ligand 1 expression in human cancer cell lines in two-dimensional and three-dimensional cell culture systems

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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 (Figure 1) (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.

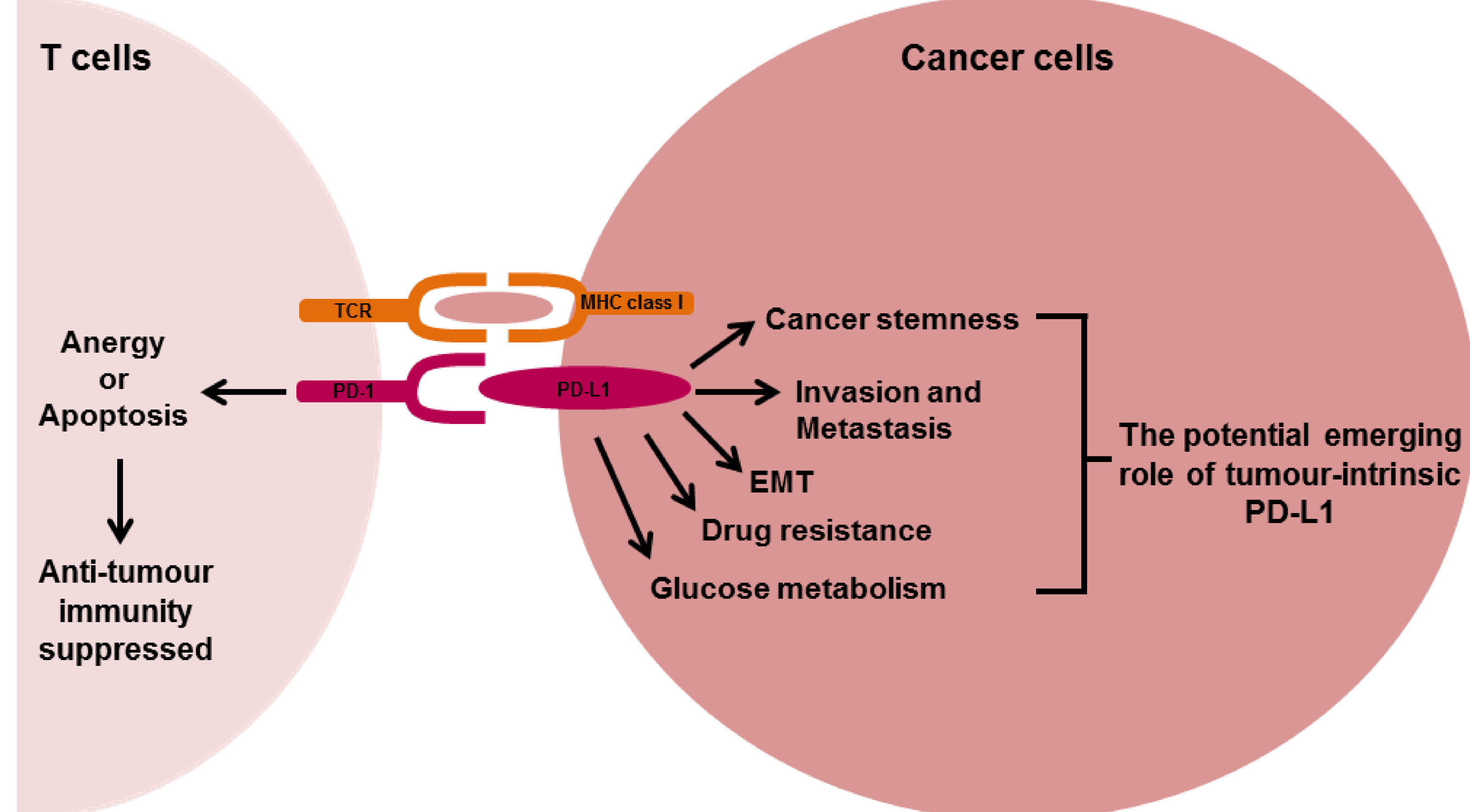

Figure 1. PD-L1/PD-1 signalling axis in cancer initiation and progression. PD-L1 binding to its receptor, PD-1 on T cells inhibits T cell activation by interfering with the T cell receptor (TCR) signal transduction via the recruitment of SHP1 and SHP2, leading to T cell anergy or apoptosis, supressing anti-tumour immunity. PD-1 is also expressed by other immune cells such as NK cells and B cells. In select cancer types, there is a potential emerging role of PD-L1 signalling in cancer cells. PD-L1 signalling in some cancer cells has been shown to promote cancer cell stemness, epithelial to mesenchymal transition (EMT), invasion, metastasis, drug resistance and regulate glucose metabolism (Dong *et al.,* 2018).

Aims and Hypothesis

- This research aimed to determine whether the cell surface expression of PD-L1 was altered in human cancer cell lines when cultured in 3D as opposed to standard 2D cell culture.
- It was hypothesised that human breast, prostate and colorectal cancer cell lines would express altered levels of cell surface PD-L1 in 3D cell culture compared to their 2D counterparts.

Methods

Cell culture Human cancer cell lines were cultured in RPMI (MDA-MB-231, MCF-7, LNCaP and PC3) or DMEM (SW480 and SW620) media supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were cultured in 2D and 3D; two well-established 3D cell culture systems were utilised (Figure 2). All cell lines were purchased from ATCC, frequently tested for mycoplasma and were below passage 30 for all experiments.

Cell viability in 3D spheroids At specific time points a single alginate bead or spheroid for each cell line was harvested, 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.

Cell surface staining Human cancer cell lines growing in 3D spheroids and their monolayer counterparts were stained with fluorescently labelled anti-human PD-L1 and matched isotype control. PD-L1 cell surface expression was assessed using flow cytometry and data was analysed using FlowJo software. The median fluorescent intensity (MFI) was normalised relative to the isotype control and appropriate gating of single and live cells was carried out for each experiment.

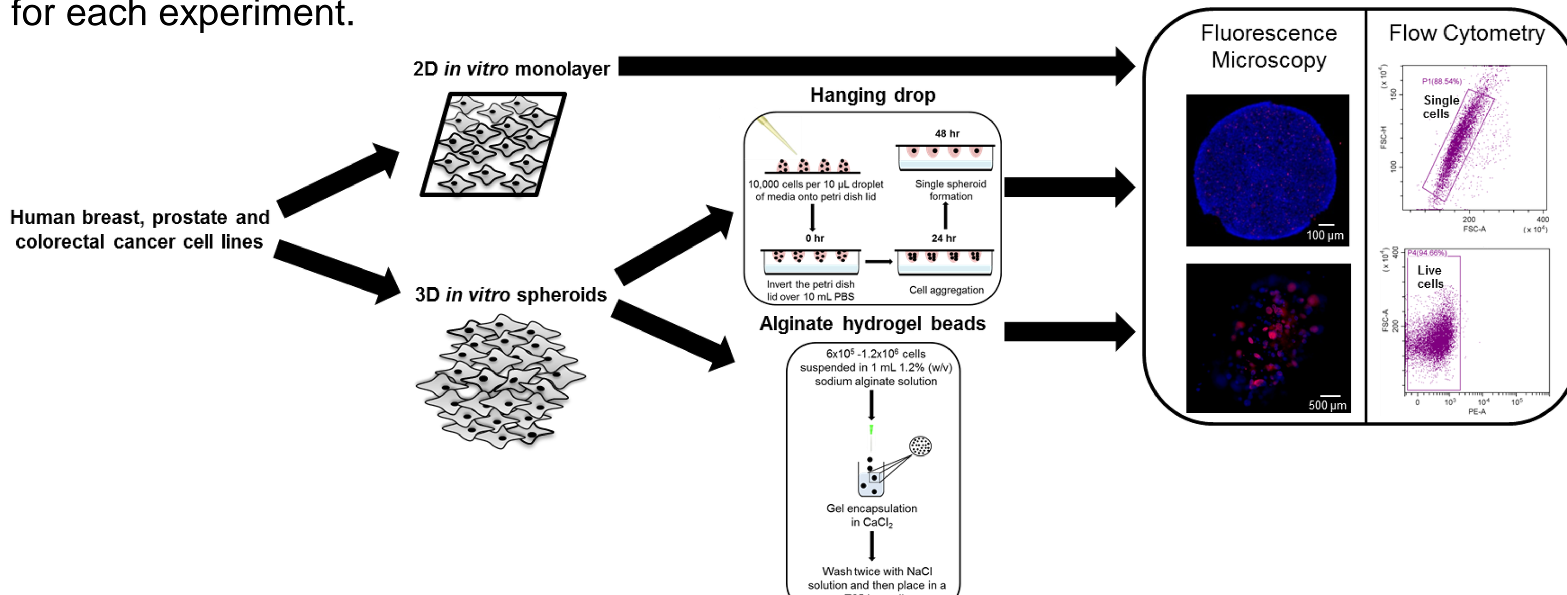

Figure 2. A schematic diagram of the work flow applied to investigate human cancer cell PD-L1 cell surface expression in 2D versus 3D using two well-established 3D cell culture systems. Human breast, prostate and colorectal cancer cell lines were grown in 2D and 3D cell culture for investigating PD-L1 cell surface expression. For 3D cell culture, the hanging drop method and alginate hydrogel beads were utilised which facilitate the formation of spheroids that display heterogeneous population of cells resembling that found in the tumour microenvironment. Spheroids generated were harvested for microscopic and flow cytometric analysis at day 3 from hanging drop and day 3, day 6 and day 10 from alginate hydrogel beads.

Results

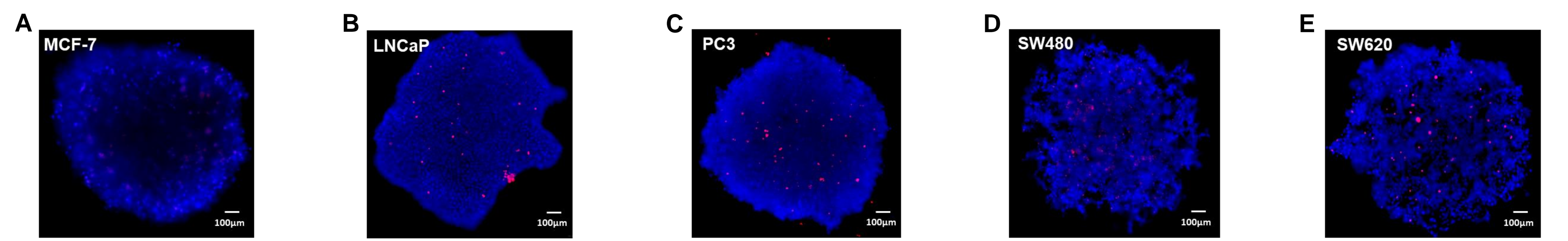

Figure 3. Human breast, prostate and colorectal cancer cell lines form viable and intact spheroids. Single spheroids generated by hanging drop were harvested at day 3 for viability assessment using Hoechst 33342/PI staining. MCF-7 **(A)**, LNCaP **(B)**, PC3 **(C)**, SW480 **(D)** and SW620 **(E)** cancer cells form viable and intact spheroids at day 3 in hanging drop. MDA-MB-231 cancer cells disaggregate upon harvesting from the petri dish lid. Day 3 viability for MDA-MB-231 cancer cells was assessed by flow cytometry. Scale bar represents 100 µm.

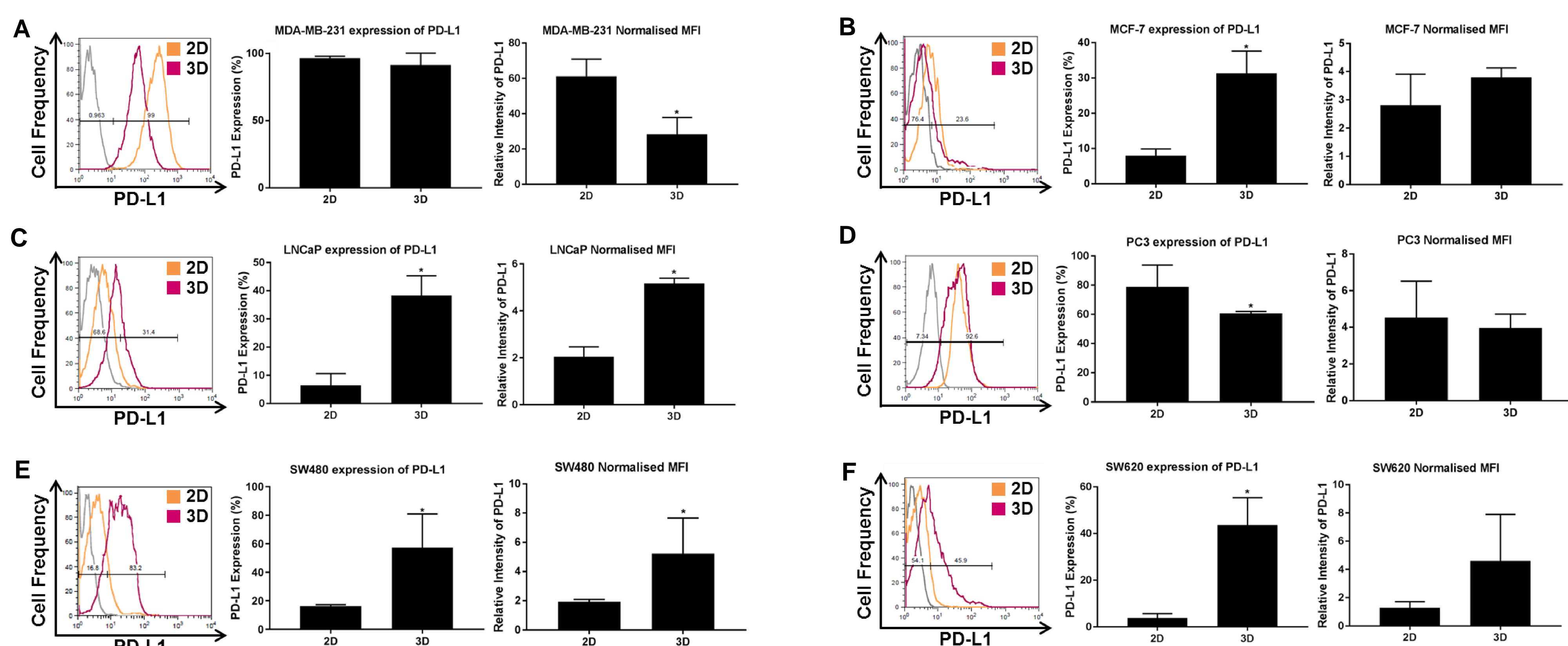

Figure 4. Human breast, prostate and colorectal cancer cell spheroids generated using the hanging drop method display altered PD-L1 expression when compared to their 2D counterparts at day 3. MDA-MB-231 cancer cells exhibit a significant decrease in the level of PD-L1 expression at day 3 **(A)**. MCF-7 **(B)**, LNCaP **(C)**, SW480 **(E)** and SW620 **(F)** cancer cells display a significant increase in PD-L1 expression at day 3. LNCaP and SW480 also display an increase in the level of PD-L1 expression at day 3. PC3 cancer cells show a significant decrease in PD-L1 but display no change in the level of PD-L1 expression at day 3 **(D)**. Representative flow cytometry plots are displayed for each cell line showing the isotype control (grey) relative to the PD-L1 positive populations for cells grown in 2D (orange) and 3D (pink). The PD-L1 percentage expression is graphed alongside the normalised MFI. Data is presented as mean ± SD, n=3 independent experiments each with 3 technical repeats, Mann-Whitney U test, *P<0.05.

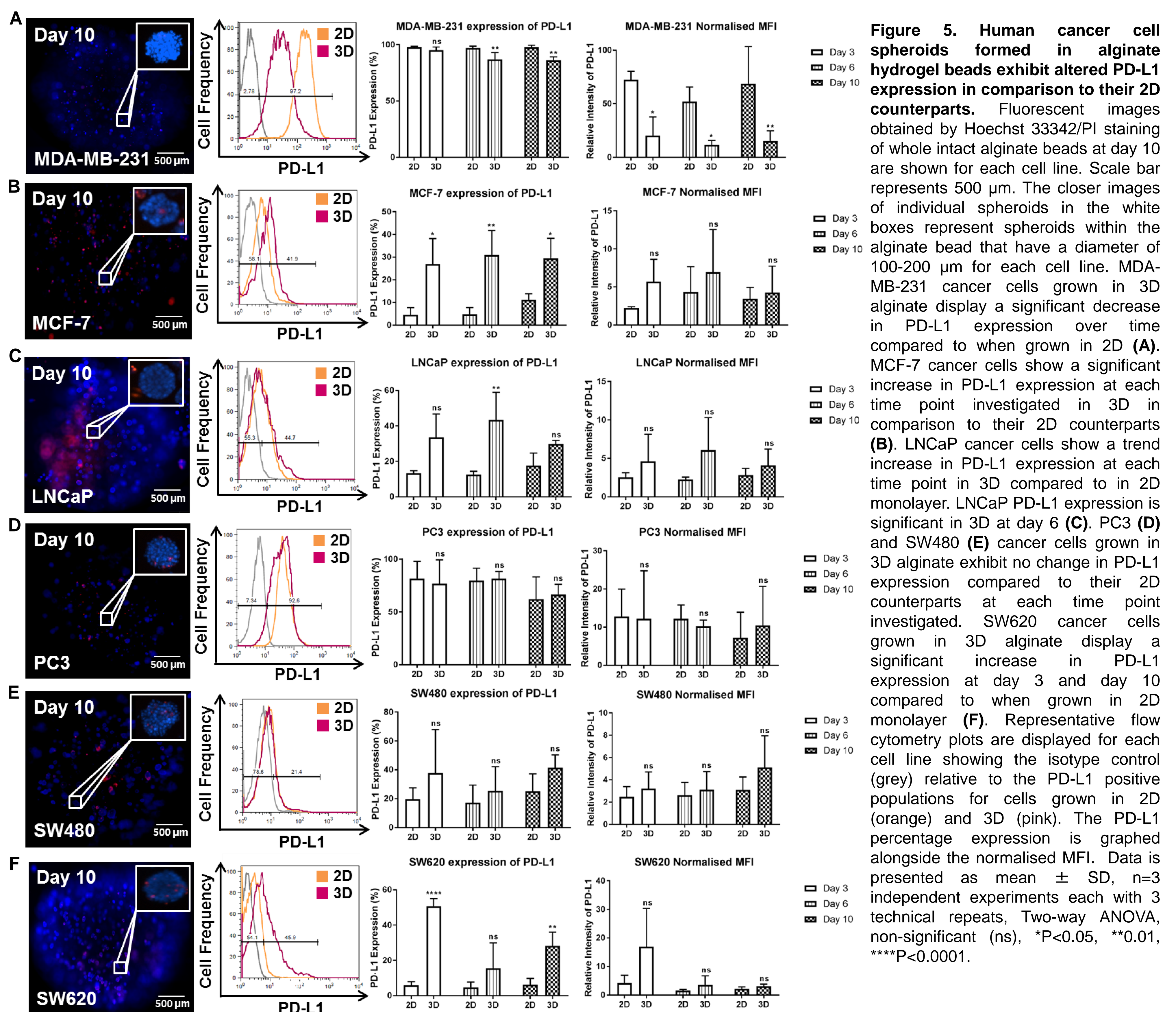

Figure 5. Human cancer cell spheroids formed in alginate hydrogel beads exhibit altered PD-L1 expression in comparison to their 2D counterparts. Fluorescent images obtained by Hoechst 33342/PI staining of whole intact alginate beads at day 10 are shown for each cell line. Scale bar represents 500 µm. The closer images of individual spheroids 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 alginate display a significant decrease in PD-L1 expression over time compared to when grown in 2D **(A)**. MCF-7 cancer cells show a significant increase in PD-L1 expression at each time point investigated in 3D in comparison to their 2D counterparts **(B)**. LNCaP cancer cells show a trend increase in PD-L1 expression at each time point in 3D compared to in 2D monolayer. LNCaP PD-L1 expression is significant in 3D at day 6 **(C)**. PC3 **(D)** and SW480 **(E)** cancer cells grown in 3D alginate exhibit no change in PD-L1 expression compared to their 2D counterparts at each time point investigated. SW620 cancer cells grown in 3D alginate display a significant increase in PD-L1 expression at day 3 and day 10 compared to when grown in 2D monolayer **(F)**. Representative flow cytometry plots are displayed for each cell line showing the isotype control (grey) relative to the PD-L1 positive populations for cells grown in 2D (orange) and 3D (pink). The PD-L1 percentage expression is graphed alongside the normalised MFI. Data is presented as mean ± SD, n=3 independent experiments each with 3 technical repeats, Two-way ANOVA, non-significant (ns), *P<0.05, **0.01, ****P<0.0001.

Conclusion and Future Direction

Utilising 3D cell culture systems including the hanging drop method and alginate hydrogel beads, we have found that PD-L1 cell surface expression was altered consistently in both 3D cell culture methods in all the human cancer cell lines investigated with the exception of PC3 and SW480 cells in alginate when compared to their 2D counterparts. The level of expression of PD-L1 by cancer cells in 3D is more likely to mimic that of an *in vivo* human tumour microenvironment than standard 2D cell culture, and may better able the investigation of the tumour-intrinsic role of PD-L1, cancer cell-immune cell interactions and cancer cells response to PD-L1 targeted treatment.

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