Adoption and Validation of *In Vitro* Co-Culture Assays to support Immuno-Oncology Research and Drug Discovery

Katie Hudson, Gareth Edwards, Lara De Tomasi, Anushuya Tamang and Shailendra Singh

Cellomatics Biosciences Ltd, 10 Colwick Quays Business Park, Nottingham, UK

Immuno-Oncology *In Vitro* Assays To Support Early-Stage Drug Discovery

Small molecule and Biologic high throughout screening → **Mechanism of action studies** → **Primary cells or cell lines in 2D monolayer or 3D spheroids** → **Cytotoxic T cell killing, ADCP and ADCC, Macrophage differentiation and polarisation, Immune cell proliferation, Immune checkpoints** → **Flow cytometry, Cytokine release, Live/Fixed cell imaging, Proliferation assays, Luciferase bioassays**

Cytotoxic T Cell Killing Assay using 3D Cancer Spheroids

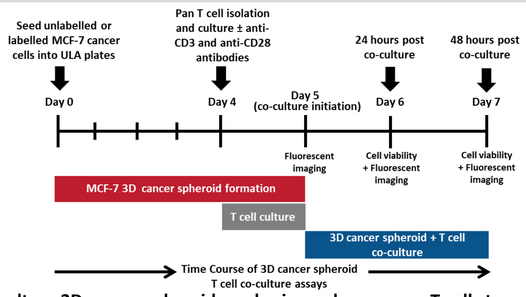


Figure 1. Schematic workflow applied to co-culture 3D cancer spheroids and primary human pan T cells to assess T cell-mediating killing of cancer cells. Unlabelled or labelled MCF-7 cancer cells were seeded into ultra low attachment (ULA) plates and cultured for 5 days prior to co-culture to facilitate 3D spheroid formation. Primary human Pan T cells were freshly isolated from healthy donor blood and cultured for 24 hours in appropriate media with or without anti-CD3 and anti-CD28 stimulation antibodies prior to co-culture. Pan T cells were harvested and counted before being seeded into the ULA plates either alone or in co-culture with the MCF-7 3D spheroids at 10:1 and 20:1 Effector:Target ratios. T cell-mediated cytotoxicity and infiltration was then measured by performing a cell viability assay or fluorescent imaging, respectively.

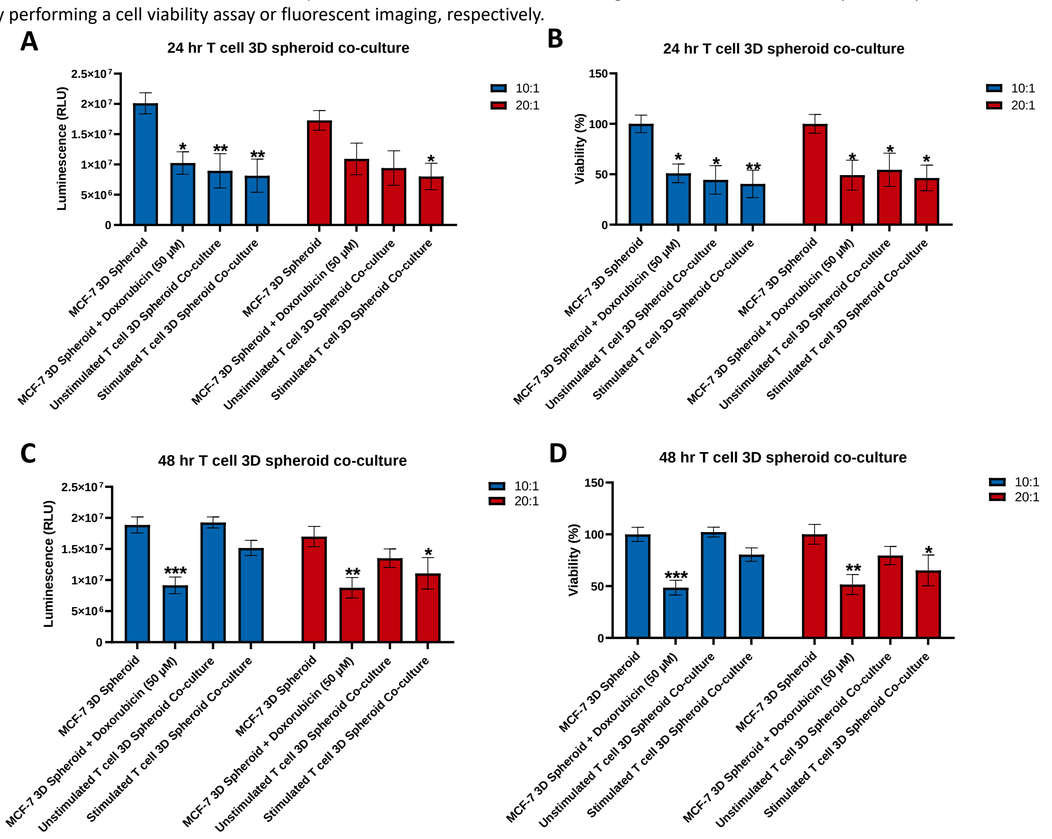


Figure 2. T cell-mediated cytotoxicity towards MCF-7 3D cancer spheroids. T cell-mediated cytotoxicity towards MCF-7 3D spheroids was measured at **(A, B)** 24 hours and **(C, D)** 48 hours post co-culture using the CellTiter-Glo® 3D Cell Viability Assay and by reading the luminescent signal on the GloMax® Discover Microplate Reader. Doxorubicin (50 µM), known to induce MCF-7 cancer cell death, was used in this assay as a positive control for cancer cell death. At 24 hours, Doxorubicin, unstimulated and stimulated Pan T cells at both ratios induced significant cell death in MCF-7 3D spheroids compared to the untreated 3D spheroids. Similarly, at 48 hours, Doxorubicin and stimulated Pan T cells at 20:1 ratio induced significant cancer cell death compared to untreated 3D spheroids. To specifically detect cancer cell death, the luminescent values from unstimulated and stimulated Pan T cells alone were subtracted from the luminescent values of co-cultures. Data were represented as the mean ± SEM of 3 independent experiments each with 3 replicates.

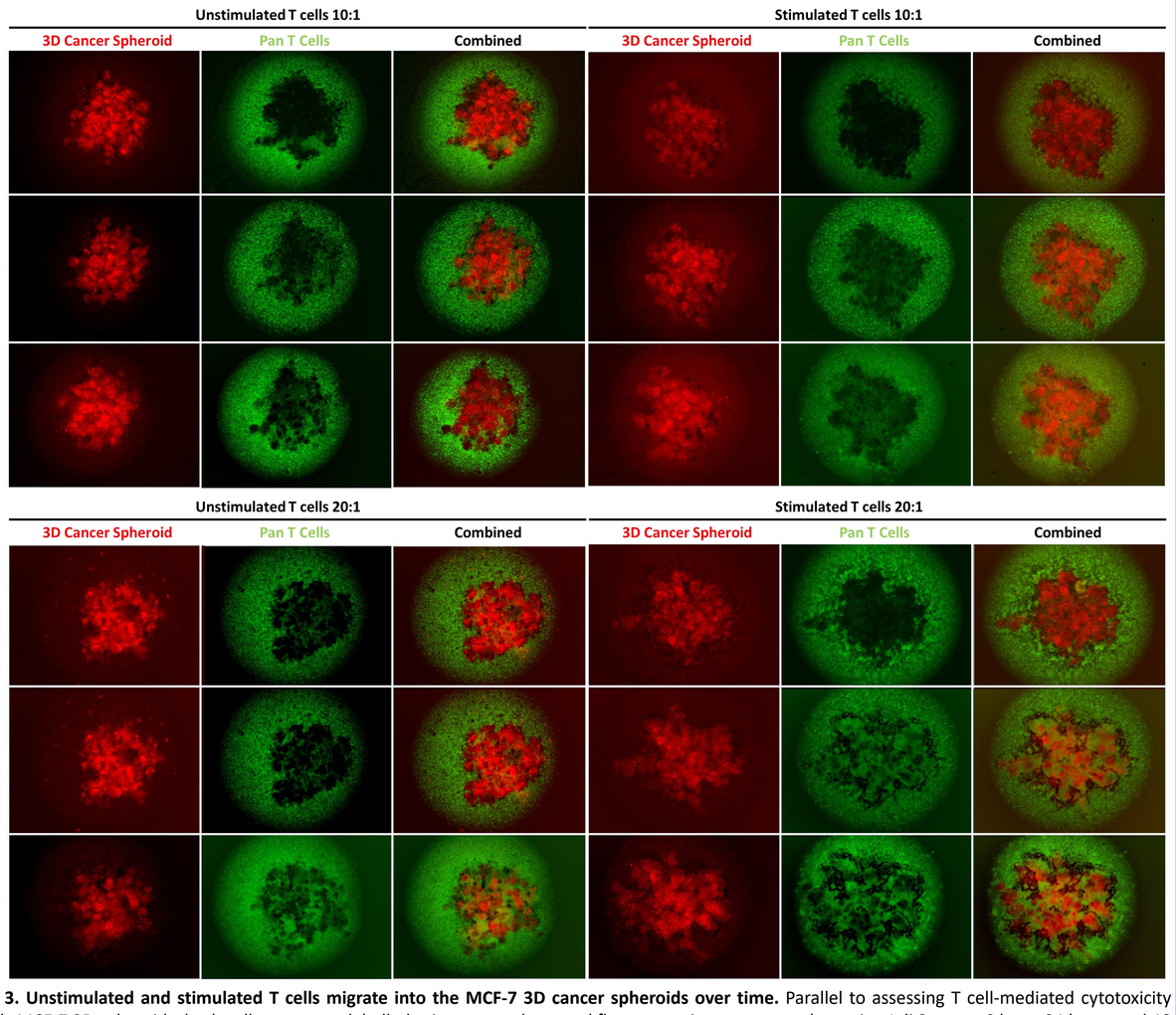


Figure 3. Unstimulated and stimulated T cells migrate into the MCF-7 3D cancer spheroids over time. Parallel to assessing T cell-mediated cytotoxicity towards MCF-7 3D spheroids, both cell types were labelled prior to co-culture and fluorescent images were taken using Juli Stage at 0 hour, 24 hours and 48 hours post-culture to assess T cell migration into the MCF-7 3D spheroids. Unstimulated and stimulated Pan T cells (green) at 10:1 (top panel) and 20:1 (bottom panel) ratios show evidence of migration into the MCF-7 3D spheroids (red). Pan T cells seeded at 20:1 ratio displays greater infiltration into the MCF-7 3D spheroids compared to those seeded at 10:1 ratio. Considering the imaging and cell viability data together may suggest that the degree of T cell infiltration into the MCF-7 3D spheroids may not be a determining factor for the T cells to elicit their cytotoxic effects towards cancer cells and that T cells may be able to induce cancer cell death from the periphery of the spheroid. Images are representative of n=3 replicates.

Macrophage Differentiation/Polarisation and Characterisation

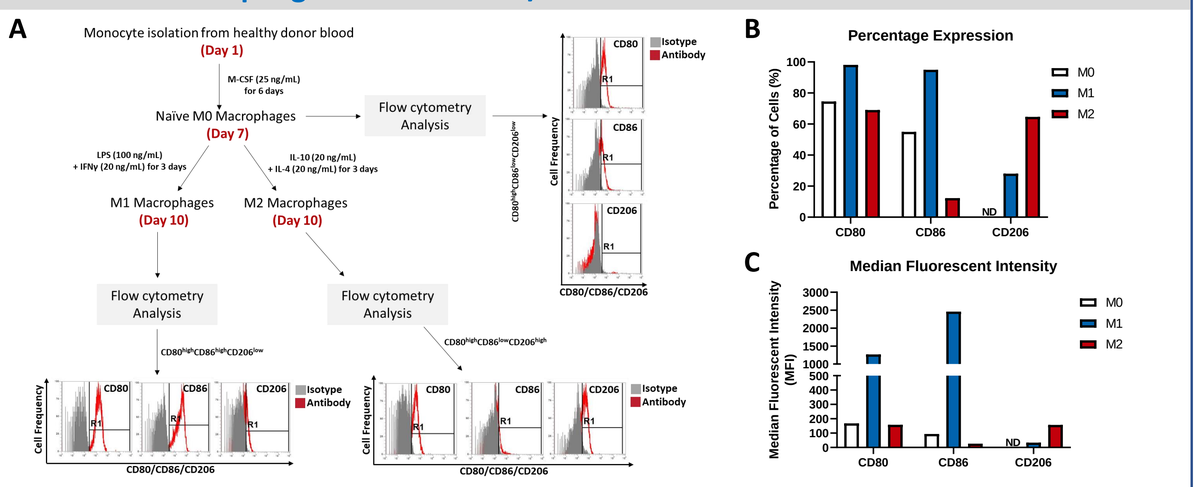
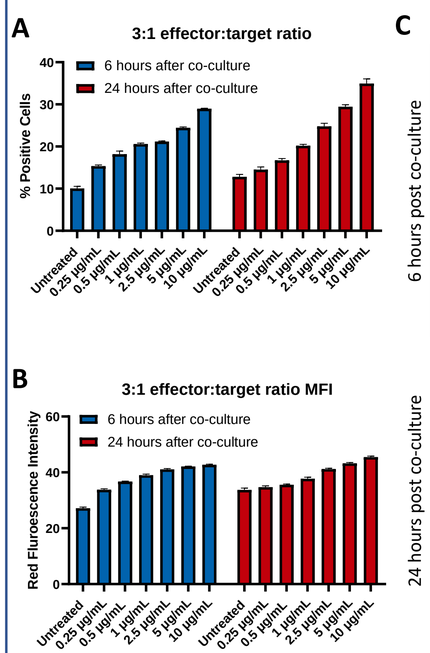


Figure 4. Schematic work flow to generate M0, M1 and M2 type macrophages from primary human monocytes. (A) The work flow applied to generate different macrophage phenotypes derived from primary human monocytes. **(B)** The percentage positivity is graphed alongside **(C)** the median fluorescent intensity (MFI) of CD80, CD86 and CD206 expression for M0, M1 and M2 primary-derived human macrophages. Data are representative of a single donor. Data was normalised to the appropriate isotype controls. ND = Not detectable.

ADCP of Cancer Cells by Naïve Human Primary Macrophages



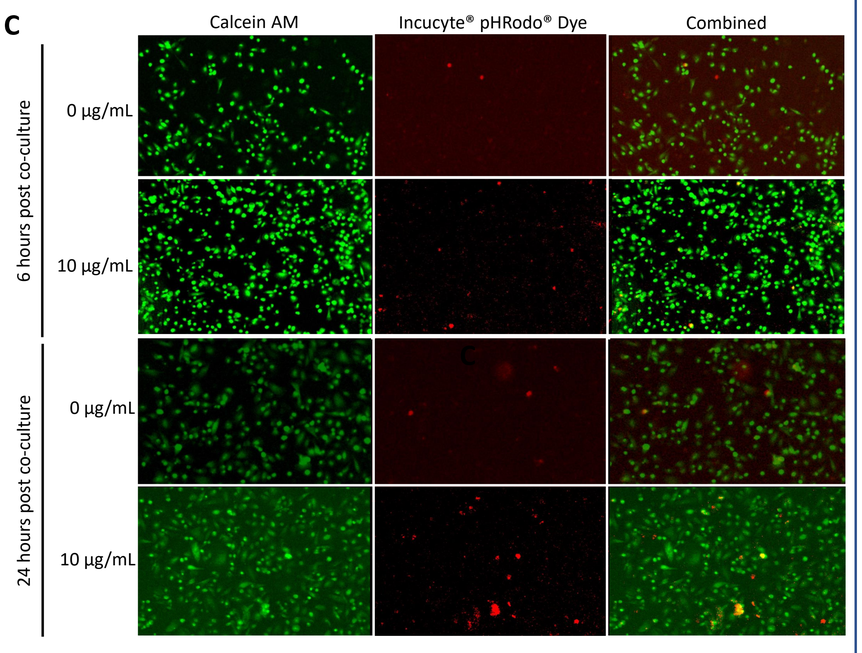


Figure 5. The phagocytic activity of primary naïve macrophages towards MCF-7 breast cancer cells treated ± HER2 targeting monoclonal antibody Trastuzumab. Primary monocytes were isolated from healthy donor whole blood and differentiated into naïve macrophages which were stained with Calcein AM prior to co-culture with MCF-7 breast cancer cells at an effector to target ratio of 3:1. MCF-7 cells were treated with different concentrations of Trastuzumab for 1 hour and labelled with the Incucyte® phRodo® dye prior to co-culture. ADCP activity was assessed at 6 hours and 24 hours post co-culture through fluorescent imaging using JuLI Stage. **(A)** The percentage of target cells positive for phagocytosis was determined by dividing the number of target cells (MCF-7) that have been phagocytosed relative to the total number of target cells (MCF-7) seeded at the start of co-culture. **(B)** The median fluorescent intensity of red staining was also graphed and is indicative of phagocytosis. **(C)** Representative images are shown for co-cultures treated with 0 and 10 µg/mL of Trastuzumab at both 6 hours and 24 hours post co-culture. Data were represented as the mean ± SEM of 3 replicates.

PD-1 blockade on activated T cells within the PBMC population

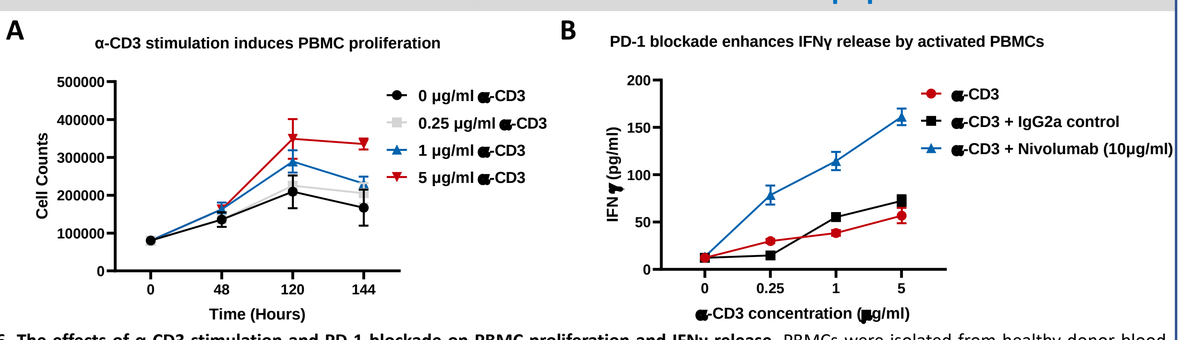


Figure 6. The effects of α-CD3 stimulation and PD-1 blockade on PBMC proliferation and IFNγ release. PBMCs were isolated from healthy donor blood and cultured in the presence α-CD3 (0, 0.25, 1 and 5 µg/mL) for up to 6 days. **(A)** PBMC proliferation was induced by α-CD3 stimulation at the higher concentrations investigated. **(B)** α-CD3 stimulated PBMCs were treated with 10 µg/mL Nivolumab and IFNγ release was measured after 96 hours. PD-1 blockade enhanced IFNγ release by α-CD3 stimulated PBMCs in a dose dependent manner. Data were represented as the mean ± SEM of 3 replicates.

Validation of the PD-1/PD-L1 luciferase bioassay using PD-L1+ CHO-K1 cells and PD-1+ Jurkat T cells

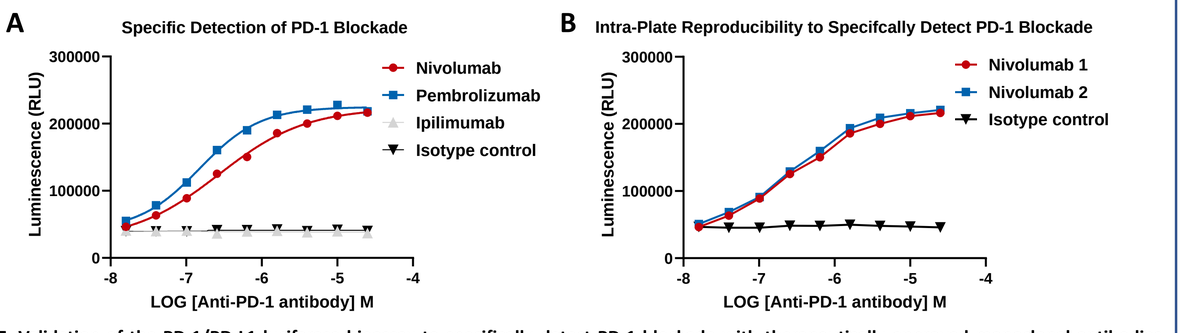


Figure 7. Validation of the PD-1/PD-L1 luciferase bioassay to specifically detect PD-1 blockade with therapeutically approved monoclonal antibodies, Nivolumab and Pembrolizumab. The PD-1/PD-L1 luciferase bioassay allows detection of the inhibitory activity of PD-1 and PD-L1 blocking antibodies as quantified by increases in luminescence. PD-L1 expressing CHO-K1 target cells were plated and treated with increasing concentrations of PD-1 blocking antibodies and PD-1 expressing Jurkat effector cells before the luminescence was measured using the Bio-Glo™ Luciferase Reagent and GloMax® Discover Microplate Reader. **(A)** Validation of this assay was performed using Nivolumab and Pembrolizumab which specifically blocks PD-1 on effector cells and thus PD-1/PD-L1 interaction as observed via the increase in luminescent signal with increasing concentrations of the PD-1 target antibodies. An isotype control and Ipilimumab, an anti-CTLA4 monoclonal antibody was used to verify the specificity of assay for detection of PD-1/PD-L1 blockade. **(B)** A high degree of intra-plate reproducibility was observed for the Nivolumab inhibitory response.

CELLOMATICS BIOSCIENCES LTD.

10 Colwick Quays Business Park, Road No 2, Colwick, Nottingham, NG4 2JY, UK

T: +44 (0) 115 865 4101 E: info@cellomaticsbio.com www.cellomaticsbio.com