VITVO™: A Novel 3D In Vitro System For Functional Prediction Of Patient Anti-tumor Immune Response To Checkpoint Inhibitors

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Introduction

- Immunotherapy has become a crucial modality to fight cancer leading to the reactivation of the tumor-suppressed immune system[1]. Among immunotherapy agents, **checkpoint inhibitors represent a promising approach to the treatment of several solid tumors, such as lung cancer[2]**. Cancer immune checkpoint therapy is based on targeting regular pathways in T cells to enhance anti-cancer immune responses, instead of bringing direct cytotoxic effects on tumor cells.
- Immune checkpoint blockade with PD-1/programmed cell death ligand 1 (PD-L1) inhibitors has thus become part of the standard-of-care treatment option for patients with advanced stage NSCLC. The interaction between PD-1 and its ligand PD-L1, plays a crucial regulatory role in the human immune system by inhibiting the body's immune response to foreign antigens. Therefore, **many cancer cell types express PD-L1 and thereby can activate PD-1/PD-L1 signaling, thus enabling these tumors to evade immune recognition.** Precision therapies that focus on the PD-1/PD-L1 pathway can offer a novel treatment avenue to some patients with cancer[3].
- PD-L1 expression is biomarker for the prediction of response to anti-PD-(L)1 immunotherapies[4]. However, **the predictive value of PD-L1 expression or immunotherapy are currently debating and challenging** and, although many researchers have worked on this issue, it is not yet solved. Different detecting methods with combined biomarkers may provide new strategies that can help us select patients for cancer immunotherapy.
- Here we present **an innovative strategy based on a novel 3D small bioreactor called VITVO™,[5], conceived to rebuild in vitro the tumor microenvironment.**
- Using this platform primary cells harvested from human lung cancer specimens have been evaluated in order to predict the patient specific anti-tumor immunity of tumor infiltrating lymphocytes (TILs) triggered by checkpoint inhibitor, Nivolumab.

Material and Methods

- **Patients and samples.** The study was approved by the Ethical and Institutional Review Board at the University Hospital of Modena and was carried out in accordance with the relevant guidelines and regulations. Tumor specimens were obtained after signed informed consent from patients who underwent surgery at the Thoracic Surgery Division (University-Hospital of Modena). Tumor tissues were dissociated into single cells with Human Tumor Dissociation Kit (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) using GentleMACS Octo Dissociators with heaters protocol's (Miltenyi Biotec).
- **FACS analysis.** After tumor tissue digestion cells were counted, seeded in fluorescence-activated cell sorting (FACS) analysis polypropylene tubes (1x10^6/tube; VWR, Milan, Italy) and incubated in blocking buffer (100µl each 1x10^6 MSC) containing Dulbecco's modified Eagle medium (DMEM), 10% fetal bovine serum (FBS), 0.1M sodium azide and 66.6 mh/ml human immunoglobulin G (Sigma, Steinheim, Germany) for 20 minutes on ice. Cells were subsequently stained for 30 minutes on ice with primary antibodies in PBS with 0.1% bovine serum albumin (BSA, Sigma) and analyzed with FACS ARIA III (BD Bioscience, Franklin Lakes, NJ, USA). The following monoclonal antibodies were introduced: CD3-PE, CD8-APC, CD4-FITC; 7-amino-actinomycin-D (7AAD)-staining (ViaProbe, BD Bioscience; 20µl/1x10^6 cells, according to the manufacturer's instructions) was evaluated by flow cytometry to detect apoptosis.
- **VITVO™ 3D model.** VITVO™ was first primed with media alone to ensure the complete wetting of 3D matrix. Afterwards 2x10^6 primary cells (unfractioned population) were resuspended in 1,4 ml of culture media (RPMI, 10% FBS, 1% glutamine) and injected into the system by a 2.5ml syringe.
- **Drug treatment.** Nivolumab (Opdivo, Bristol-Myers Squibb, New York, USA) was used at a final concentration of 40 µg/ml and was added in VITVO™ within culture media as a single dose.
- **In VITVO™ viability and cytotoxicity assessment.** Cell vibility was evaluated by Real Time-GLO™ MT cell viability assay (Promega, Madison, WI, USA). The fluorescent based Live/Dead® assay (Invitrogen, ThermoFisher Scientific) was used to visualize into the 3D matrix tumor cell death by microscopy.
- **Statistics.** All measurements were performed in triplicate. Data have been analyzed using Microsoft Excel 2016 (Microsoft Corporation) and are expressed as mean values ±SD or ±SEM. Un-paired two-tailed Student's test was used considering $p \leq 0.05$ as statistically significant.

Results

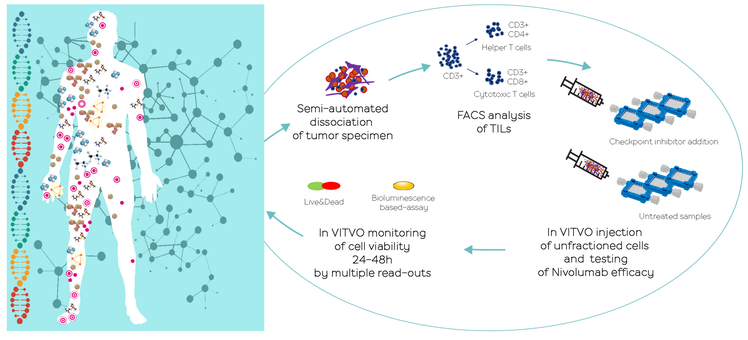

Figure 1. Experimental approach overview

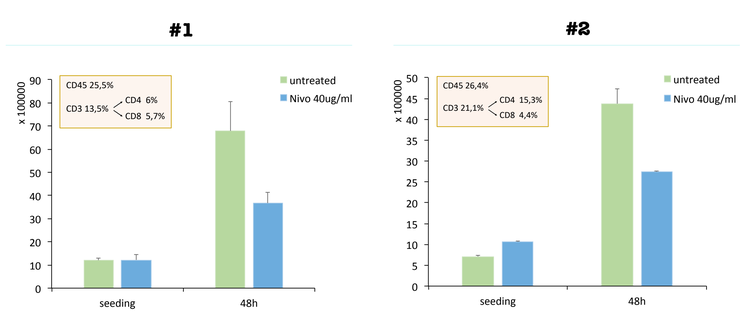

Figure 2. Tumor viability evaluation by Real Time-GLO™. #1 and #2 are samples from different donors. FACS analysis of tumor infiltrating lymphocytes is shown in yellow inset panels.

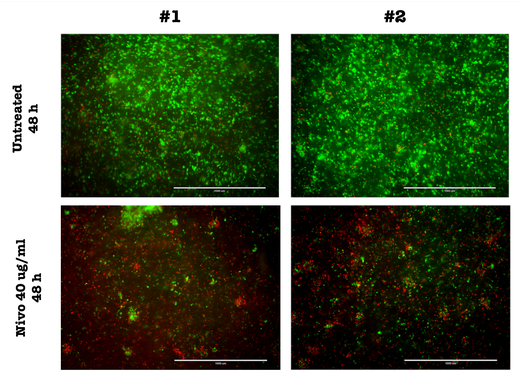

Figure 3. Live/Dead® staining (Live cells in green; Dead cells in red) and imaging by fluorescence microscope (EVOS FL AUTO, Invitrogen).4X magnification with super apochromatic objective (Olympus).

Conclusions

- Immunotherapy is an increasing treatment option for cancer patients . However, **only a subset of patients responds to currently approved immune checkpoint inhibitors.** This is mostly due to the inability of the cytotoxic T lymphocytes to react. **How to predict this event is unclear and still associated with histo-pathological readouts.** Therefore **there is a need to generate novel functional assays** to predict checkpoint inhibitors action as fundamental aspect to generate more targeted approaches associated with a better anti-cancer action with ethical and pharmaco-economics implications.
- Our tool aims to recreate a three-dimensional (3D) tumor microenvironment in order to study the multicellular interactions that direct the immune response against cancer, which is impossible to study in 2D culture systems. Cells dissociated from NSCLC obtained after thoracic surgery were loaded in the novel **3D bioreactor VITVO™** in order to recreate the tumor microenvironment in all its component, including TILs, important players in the immune response against tumor cells.
- Our findings suggested that VITVO™ can host primary tumor cells to be rapidly introduced in a functional assay for cancer responsiveness to checkpoint inhibitors predicting the anti-tumor efficacy of Nivolumab elicited by own patient TILs activation against tumor cells.
- **This innovative functional tool, evaluating the tumor responsiveness to immunomodulatory agents, represents a useful predictive assay for cancer immunotherapy and immunoprecision medicine.**

References

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