# Interrogation and Modulation of the Immunosuppressive Activity of Human TAM-like Macrophages Using *In Vitro* Cultures

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## 1. INTRODUCTION

The tumour microenvironment (TME) supports the development of pro-tumoral macrophages. These Tumour Associated Macrophages (TAMs), resemble M2-macrophages and are characterised by an immune-suppressive phenotype. In the TME, TAMs can induce proliferation and survival of tumour cells, facilitate angiogenesis, and suppress anti-tumour immune responses via expression of co-inhibitory molecules such as PD-L1 and cytokines such as IL-10 or TGF-β1. Therefore, TAMs are a highly attractive target for innovative cancer immunotherapies. Understanding whether candidate immunotherapies can reverse TAM-mediated immune suppression or reprogram TAMs to a pro-inflammatory phenotype is key to the development of effective cancer immunotherapy. Here we outline a suite of macrophage assays that can be adopted to determine whether candidate therapeutics can reverse human M2 macrophage-mediated immune suppression and repolarise M2 macrophages.

## 2. METHODS

### Monocyte-derived Macrophages:

Blood from healthy human volunteers is used to isolate human PBMC populations. Human PBMCderived monocytes are differentiated with M-CSF to skew towards a CD163+ M2 phenotype. The *in vitro* polarising regimen can be tailored towards alternate M2 subsets using cytokine cocktails. M2 macrophages are stimulated with innate immune triggers, or are co-cultured with αCD3-stimulated PBMCs. The potential of small molecule drugs and/or biologics to reverse M2 function, including immune suppression are assessed.

### Clinical material:

Tumour tissue is disaggregated by enzymatic and mechanical means to a single cell suspension, or required cell fractions are isolated using immunomagnetic separation techniques from ex vivo ascites fluid.

### Assessment of compound mediated immunomodulatory functions:

Readouts measuring clinically relevant changes in immune function and cell phenotype include: Proliferation and cell surface marker analysis (Flow cytometry) Gene expression (PCR and Quantigene Plex) Production of immunomodulatory cytokines (ELISA and multiplex).

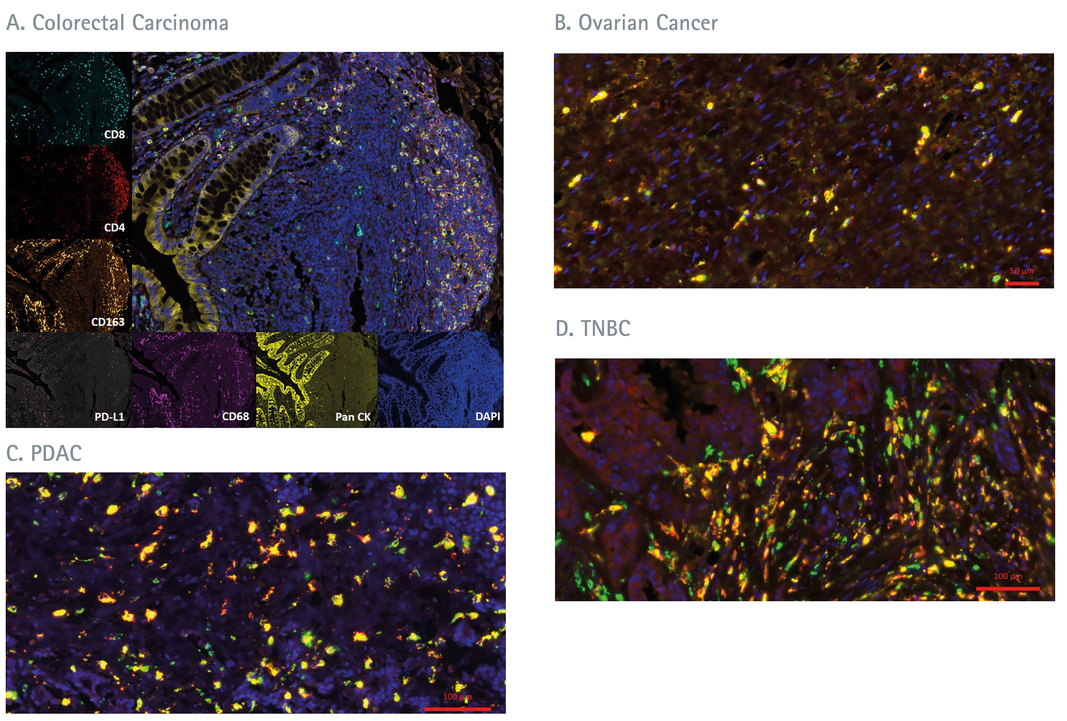


**Figure 1. Identification of TAM populations in tumour sections.**

Immunofluorescence (IF) staining performed by Aquila identified the presence of TAMs (CD68+CD163+) within sections of human tumour samples. PD-L1 colocalization with macrophage markers. CD8/CD4/CD163/ PD-L1/CD68/Pan CK protein expression in colorectal carcinoma stained by IF with Opal™ Multiplex Detection Reagents using the Vectra® Polaris™ from PerkinElmer **(A)**. CD163 (red), CD68 (green) and DAPI (blue) protein expression stained using IF in Ovarian cancer **(B)**, Pancreatic ductal adenocarcinoma (PDAC) **(C)**, and Triple negative breast cancer (TNBC) **(D)** sections. Tissue may be further analysed using RNAscope® technology.

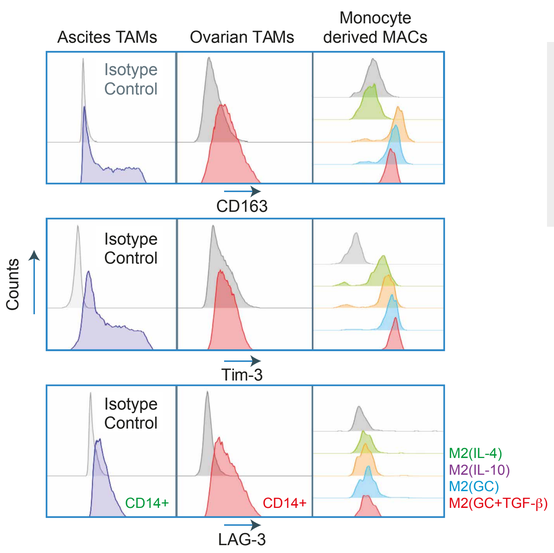


**Figure 2. Flow cytometric characterisation of tumour-derived macrophages and monocyte-derived macrophages from human PBMC.**

TAMs are immunosuppressive, resembling type 2 macrophages (M2). TAMs derived from patient ascites and ovarian tumours show expression of typical M2 marker, CD163, and checkpoint therapeutic targets, TIM-3 and LAG-3. Monocyte derived Macrophages representing the alternate M2 subsets can be used to model the suppressive functions of TAMs. These M2-like macrophages express similar surface marker expression profiles to that of TAMs derived from clinical material.

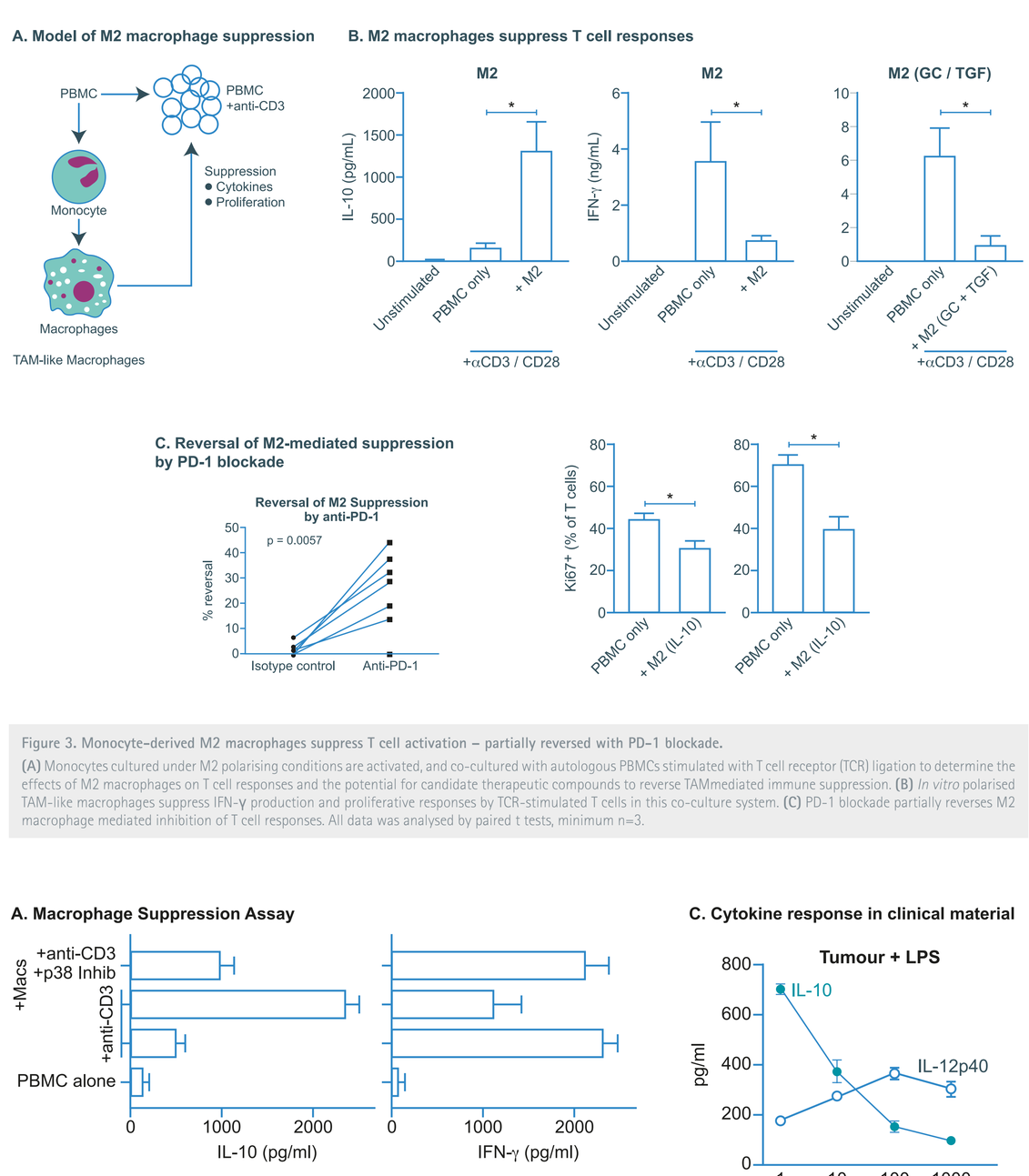


**Figure 3. Monocyte-derived M2 macrophages suppress T cell activation – partially reversed with PD-1 blockade.**

**(A)** Monocytes cultured under M2 polarising conditions are activated, and co-cultured with autologous PBMCs stimulated with T cell receptor (TCR) ligation to determine the effects of M2 macrophages on T cell responses and the potential for candidate therapeutic compounds to reverse TAMmediated immune suppression. **(B)** *In vitro* polarised TAM-like macrophages suppress IFN-γ production and proliferative responses by TCR-stimulated T cells in this co-culture system. **(C)** PD-1 blockade partially reverses M2 macrophage mediated inhibition of T cell responses. All data was analysed by paired t tests, minimum n=3.

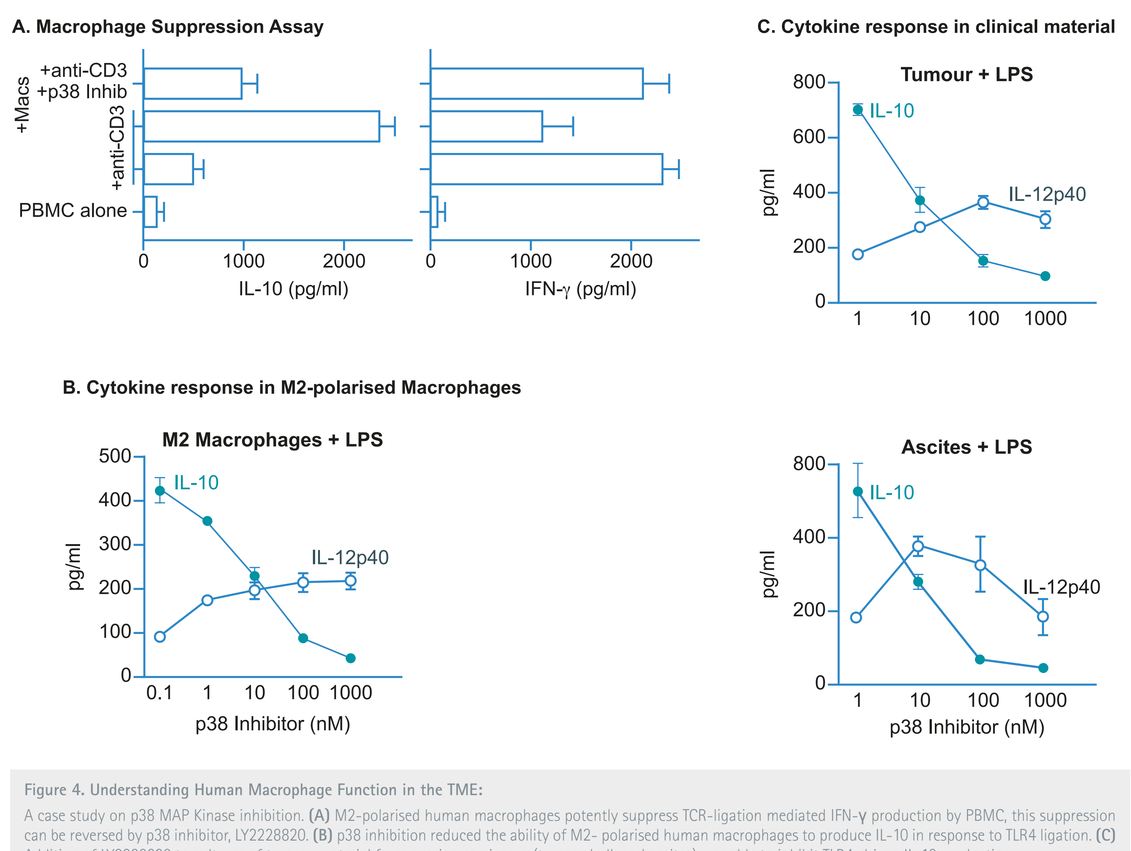


**Figure 4. Understanding Human Macrophage Function in the TME:**

A case study on p38 MAP Kinase inhibition. **(A)** M2-polarised human macrophages potently suppress TCR-ligation mediated IFN-γ production by PBMC, this suppression can be reversed by p38 inhibitor, LY2228820. **(B)** p38 inhibition reduced the ability of M2- polarised human macrophages to produce IL-10 in response to TLR4 ligation. **(C)** Addition of LY2228820 to cultures of tumour material from ovarian carcinoma (tumour bulk and ascites) was able to inhibit TLR4-driven IL-10 production.

## 3. RESULTS

Multi-spectral imaging identifies that TAMs are present within the TME associated with multiple cancer types (Figure 1). Human TAMs, such as those found in ovarian cancer, express CD163, TIM- 3, and LAG-3 (Figure 2). *In vitro* generated hum -10 production (Figure 3B) and PD-L1 signalling (Figure 3C). p38 inhibition serves as a case study to show how compound-mediated changes in functionality can be investigated using macrophages polarised to resemble TAMs (Figure 4). This switches the balance of IL-10/ IL-12 production and reduces suppressive activity (Figure 4A & B). Furthermore, access to clinical material confirms compound effects, reducing IL-10 production and elevating IL-12, indicative of a switch from immunosuppressive to immunostimulatory function in patient-derived macrophages. The suite of assays outlined here by Aquila BioMedical provide human *in vitro* and ex vivo systems to test novel compound activity upon TAM-like macrophage generation, phenotype and suppressive function. This allows selection of the most effective compounds for further investigation using patientderived immune cells. The combination of multispectral imaging, additional molecular profiling (e.g. NanoString nCounter® Analyser analysis) and the most informative functional assays on clinical material brings new opportunities to identify the most appropriate tumour type for clinical development, as well as patient stratification for immunotherapy.

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