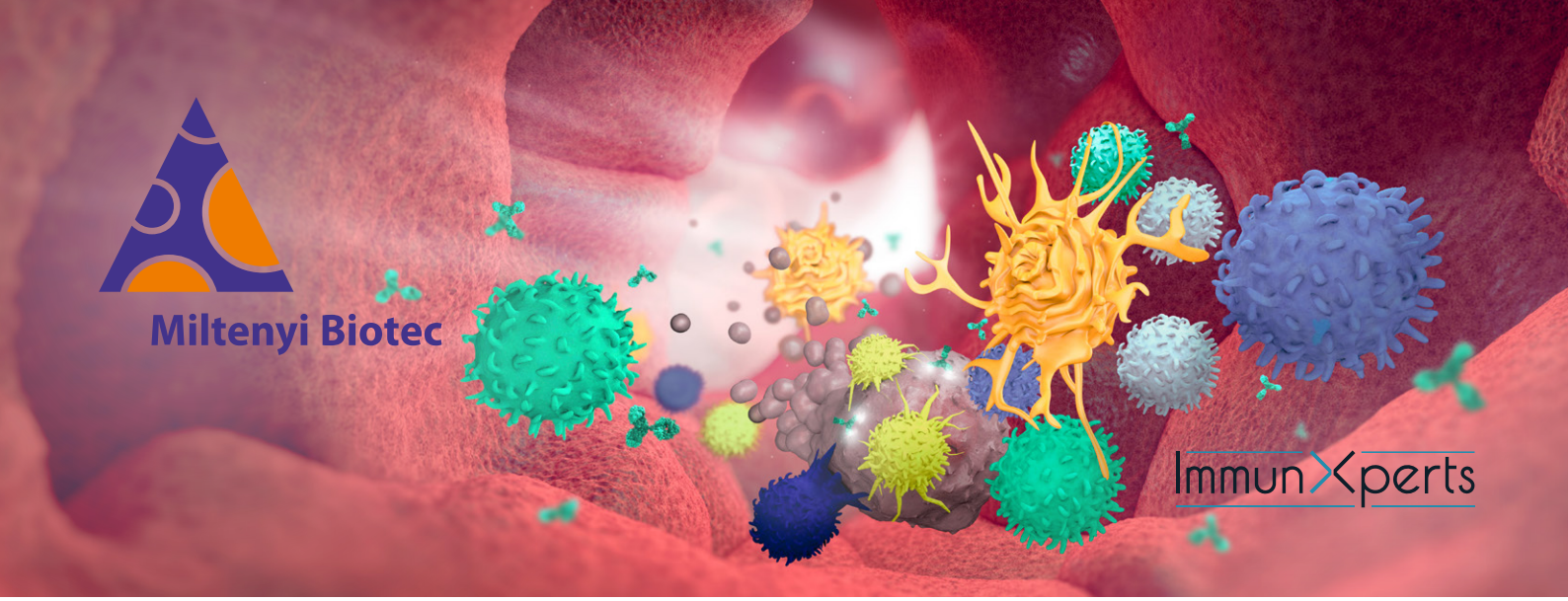


A complete macrophage suppression assay workflow making exclusive use of Miltenyi Biotec products

Authors:
Thibaut Janss, Juliette Lamy, Sofie Pattijn, Jana Schockaert

Affiliation:
info@immunxperts.com
ImmunXperts SA, Rue Auguste Piccard 48,
6041 Gosselies, Belgium

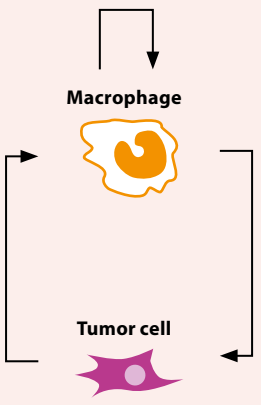
Tel: +32 71 960 133
www.immunxperts.com

In this paper, we describe how scientists from ImmunXperts used Miltenyi Biotec products to validate a macrophage suppression assay workflow. The assay includes isolation of monocyte and T cells from peripheral blood mononuclear cells (PBMCs), generation of M1/M2 macrophages with specific culturing conditions, immunophenotyping, and functional assay to confirm drug effectiveness on the suppressive capacities of the macrophages (cytokine release and proliferation) upon co-culture with CD4 T cells.

Introduction

Macrophages are a major component of the tumor microenvironment and orchestrate various aspects of immunity. Depending on their activation status, macrophages can exert dual influences on tumorigenesis. Unlike M1 macrophages, which promote a pro-inflammatory response, M2 macrophages trigger inflammation resolution and suppress T cell activation via several mechanisms. These include immune checkpoint engagement (e.g. PDL1), release of anti-inflammatory cytokines, such as IL-10 and TGFb, and metabolic activities which promote essential amino acid depletion. In most solid cancers, increased infiltration with tumor-associated macrophages (TAMs) resembling M2-macrophages has long been associated with poor patient prognosis, highlighting their value as potential diagnostic and prognostic biomarkers in cancer (fig. 1).

TAMs have increasingly become recognized as an attractive target in cancer therapy. Many preclinical studies have shown that the response to therapy can be potentiated by blocking macrophage entry into tumors[1,2], or by changing their polarization or the recruitment from an M2 to an M1 phenotype[3]. Macrophage assays to monitor the effect of candidate immunotherapeutics on TAMs are therefore very commonly performed during preclinical drug discovery.



Inflammation
Activated macrophages - LI12^{+}, MHC II^{hi}, iNOS^{+}, TNFα, CD80/CD86

Immune regulation
Immunosuppressive macrophages - ARG1^{+}, MARCO^{+}, LI10^{+}, CCL22^{+}

Angiogenesis
Angiogenic macrophages- VEGFR1^{+}, VEGF^{+}, CXCR4^{+}, TIE2^{+}

Tumor cell invasion
Invasive macrophages - WNT Signalling, EGF^{+}, MMP9, CCL3

Seeding at distant sites
Metastasis-associated macrophages - VEGFR1^{+}, VEGF^{+}, CXCR4^{+}, CCR2^{+}

Figure 1: Within the tumor microenvironment, macrophages are involved in many activities associated with tumor growth and progression, including inflammation, immune regulation, angiogenesis, invasion, and metastasis. Adapted from reference 4.

Materials and methods

PBMCs from three healthy donors were used to perform the experiment. CD14+ monocytes and CD4+ T cells were isolated from all three samples via positive and negative selection respectively with Miltenyi Biotec's MACS® MicroBeads. Macrophages were generated by culturing isolated monocytes with Miltenyi Biotec premium-grade cytokines (table 1). For M1 macrophages, monocytes were cultured with GM-CSF from D0 to D6, then GM-CSF together with IFNγ from D6 to D8. For M2 macrophages, monocytes were cultured with M-CSF from D0 to D6, then M-CSF, IL-4, IL-10, and TGFβ from D6 to D8. After that, immunophenotyping was performed using a selection of REAfinity™ Antibodies (table 2) to confirm the quality of the generated macrophages. Finally, to perform the suppression assay, isolated CD4+ T cells were labelled with proliferation dye, stimulated with a-CD3/a-CD28, and co-cultured with M1/M2 macrophages in 1:5 ratio. (Bead based activation is not recommended for this assay as they can be phagocytized by the macrophages.) Two test products, Opdivo (10 µg/mL) and Anti-PDL1 (10 µg/mL), were used to measure their effect on macrophage suppressive capacity. After five days' co-culture, supernatant was collected to measure IFNγ release, and the cells were stained with REAfinity Anti-CD4-APC Antibody, and CD4+ T cell proliferation measured using the MACSQuant® X Flow Cytometer. Data was analyzed using Flowlogic™ Software. An overview of the experimental setup is displayed in figure 2.

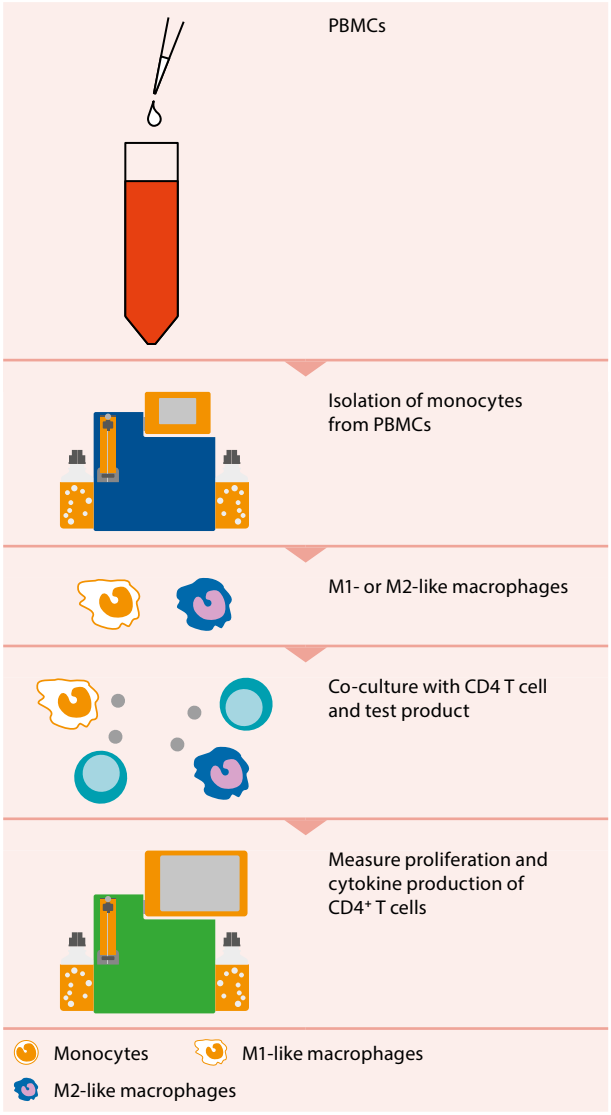


Figure 2: Schematic diagram showing experimental design.

Cytokine	Provider	Reference	Concentration used
GM-CSF	Miltenyi Biotec	130-093-866	50 ng/ml
M-CSF	Miltenyi Biotec	130-096-492	50 ng/ml
IFNγ	Miltenyi Biotec	130-096-481	20 ng/ml
IL-4	Miltenyi Biotec	130-092-921	20 ng/ml
IL-10	Miltenyi Biotec	130-093-948	20 ng/ml
TGFβ	Miltenyi Biotec	130-095-066	20 ng/ml

Table 1: Cytokines used.

Antibody	Clone name	Provider	Reference
CD163	REA812	Miltenyi Biotec	130-112-128
CD209	REA617	Miltenyi Biotec	130-120-729
CD86	REA968	Miltenyi Biotec	130-116-161
CD200R	REA725	Miltenyi Biotec	130-111-291
CD4	REA623	Miltenyi Biotec	130-113-222

Table 2: Antibodies used. Fluorochromes excited by a yellow-green or a red laser are preferable to avoid the background generated by the autofluorescence of the macrophages.

Item	Provider	Reference
CD4+ T Cell Isolation Kit, human	Miltenyi Biotec	130-096-533
Proliferation Dye	ThermoFischer	65-0842-85
CD14 MicroBeads	Miltenyi Biotec	130-050-201
MACSQuant X	Miltenyi Biotec	130-105-100
Flowlogic Software	Miltenyi Biotec	150-000-380

Table 3: Other products used.

Results

Phenotyping

Multicolor immunophenotyping results demonstrated successful polarization: M1 macrophages showed higher MFI of CD86, while M2 macrophages demonstrated higher MFI of CD163, (exclusively expressed by M2 macrophages) CD209, and CD200R protein expression in all three donor samples (fig. 3).

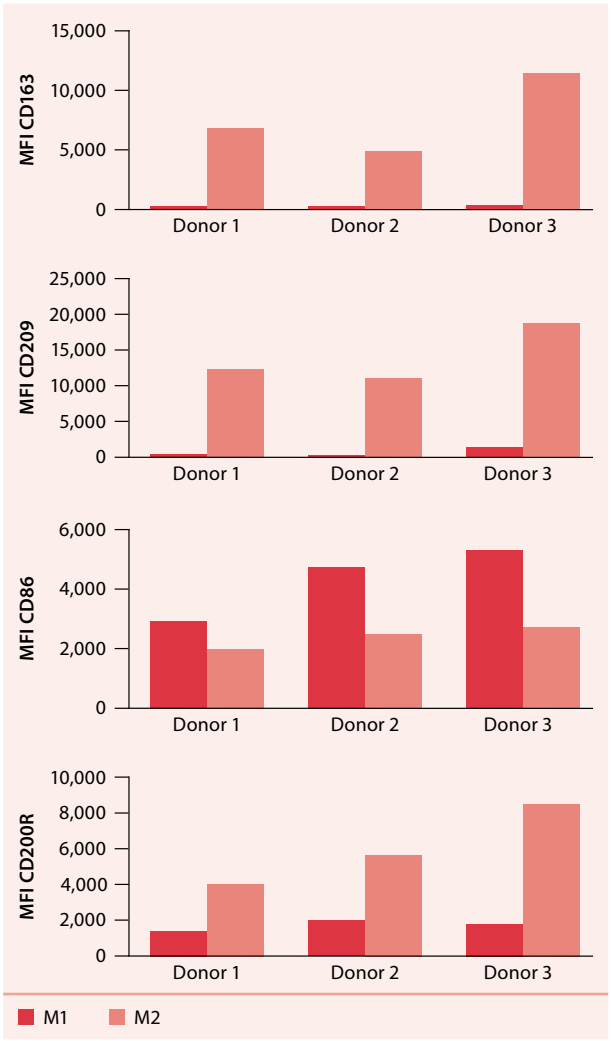


Figure 3: Phenotyping of macrophage. *Invitro*-generated macrophages were characterized by expression of CD163, CD209, CD86, and CD200R. Expression of CD163, CD209, and CD200R was higher in M2-like macrophage (light red bar). CD86 expression was higher in M1-like macrophage (dark red bar). Data represented 3 independent donor.

IFNγ secretion

In the co-culture experiment with CD4 T cells, M2-like macrophages induced a decrease in IFNγ secretion in comparison to M1 under stimulated conditions. Opdivo (anti-PD1) induced an increase of IFNγ secretion in comparison to isotype control human IgG4 in all donors. Anti-PDL1 induced an increase of IFNγ secretion in comparison to isotype control mouse IgG1 in all donors (fig. 4).

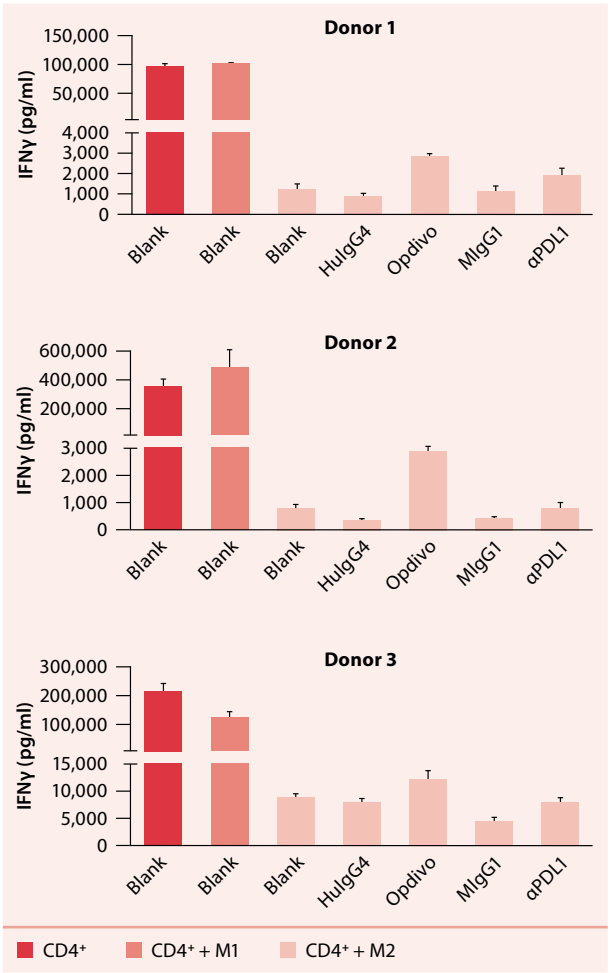


Figure 4: IFNγ secretion in co-culture assay. Comparison of secreted IFNγ among untreated CD4 T cell alone (dark red), untreated CD4 T cell + M1 macrophage (medium red) and CD4 T cells + M2 macrophages treated with different conditions (light red). Data displayed cytokine concentration in pg/ml.

Proliferation

Proliferation was observed in the cells gated on CD4 expression. M2-like macrophages induced a drastic decrease of the CD4+ T cell proliferation in comparison to M1 and CD4+ T cells alone. In donors 1 and 2, M1-like macrophages induced a slight decrease in proliferation in comparison to CD4+ T cells under activated conditions (medium exhaustion) (fig. 5).

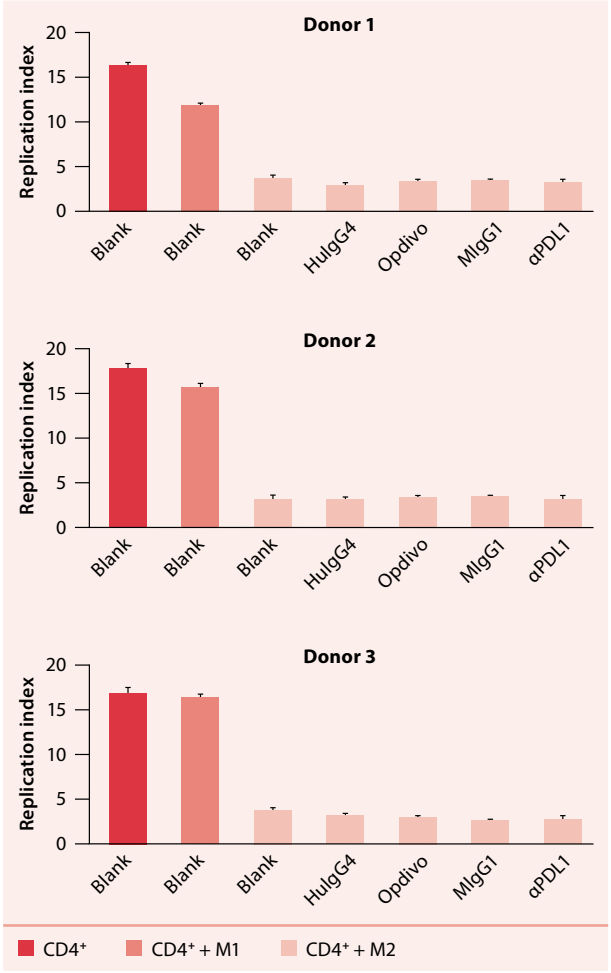


Figure 5: CD4 T cell proliferation in co-culture assay. Comparison of CD4 T cell proliferation among untreated CD4 T cell alone (dark red bar), untreated CD4 T cell + M1 macrophage (medium red bar), and CD4 T cells + M2 macrophages treated with different conditions (light red bar). Proliferation data is displayed in replication index.

Conclusion

This study demonstrates a consistent *in vitro* macrophage suppression assay using human primary immune cells, and which can be performed to analyze drug efficacy on macrophage polarization and suppression capabilities.

Miltenyi Biotec products provide a complete solution for performing macrophage suppression assays. MACS® MicroBeads allow gentle and pure separation of monocytes and CD4 T cells from donor blood, PBMCs, or other biological samples. Premium-grade cytokines allow successful and reproducible generation of M1- and M2-like macrophages from monocytes. REAfinity® Antibodies support background-free phenotyping of macrophages. Finally, the MACSQuant® X Flow Cytometer allows automated, high-throughput analysis of immunophenotyping and cell proliferation.

References

1. De Palma M. and Lewis C.E. (2013) Macrophage regulation of tumor responses to anticancer therapies. Cancer Cell. 23: 277–286.
2. Hughes R. *et al.* (2015) Perivascular M2 Macrophages Stimulate Tumor Relapse after Chemotherapy. Cancer Res. 75: 3479–3491.
3. Xu M. *et al.* (2015) Intratumoral Delivery of IL-21 Overcomes Anti-Her2/Neu Resistance through Shifting Tumor-Associated Macrophages from M2 to M1 Phenotype. J Immunol. 194:4997–5006.
4. Poh A. R. and Ernst M. (2018) Targeting Macrophages in Cancer: From Bench to Bedside. Front Oncol. 8:49

Miltenyi Biotec

Miltenyi Biotec B.V. & Co. KG | Phone +49 2204 8306-0 | Fax +49 2204 85197 | macs@miltenyibiotec.de | **www.miltenyibiotec.com**

Miltenyi Biotec provides products and services worldwide. Visit **www.miltenyibiotec.com/local** to find your nearest Miltenyi Biotec contact.

v.01