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Protocol status: Working
 We use this protocol and it's working

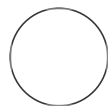
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The monocyte-derived dendritic cell (MoDC) assay – an in vitro assay for testing the immunogenicity of cellular therapies V.2

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ABSTRACT

The monocyte-derived dendritic cell (MoDC) assay can be used to test, in vitro, the immunogenicity of potential regenerative medicine cellular therapies. Similar to a mixed lymphocyte reaction (MLR), cells of interest (COI) are co-cultured with T cells, but with the addition of induced professional antigen presenting cells (APCs; moDCs) to ensure optimal T cell activation and to improve the limited sensitivity of the MLR-like assay.

Autologous CD14+ monocytes and T cells are isolated from PBMCs. Monocytes are differentiated to mature dendritic cells over 7 days, then co-cultured with T cells and cells of interest for 5 days. Recommended readouts of the assay are ELISA (for IFN γ /TNF α release) and flow cytometry (for T cell proliferation and CD25 expression).

Positive controls wells of allogeneic moDC/T cells and bead-activated T cells are utilised. Biological replicates can be run in parallel. Well-to-well variation is minimal, but samples can be run in technical duplicates if desired.

Keywords: Immunogenicity,
T cell, In vitro, monocyte-
derived DC

MATERIALS

Cell preparations:

PBMC isolation materials
MACS equipment and LS tubes
CD14+ microbeads for MACS (we use Miltenyi CD14+ microbeads)
PanT cell isolation kit for MACS (we use Miltenyi PanT cell isolation kit)
96 well tissue culture plates
Cell proliferation dye (we use eBioscience™ Cell Proliferation Dye eFluor™ 450)
Cell freezing media (FBS + 10% DMSO)

moDC differentiation

RPMI
Penicillin/streptomycin
Human AB serum
rhIL-4
rhGM-CSF
LPS
PBS

Co-culture

Cell of interest media
T cell activation beads (we use Gibco Dynabeads™ Human T-Activator CD3/CD28 for
T Cell Expansion and Activation)

Readouts

ELISA materials
Flow cytometry materials

BEFORE START INSTRUCTIONS

Fresh blood must be used each time as monocytes do not survive the freeze/thaw
process

Optimisation experiments must be performed to ensure all cell types survive without
compromise to survival, proliferation and differentiation state in the 1:1 mix of
medias (T cell/moDC media: cell of interest media)

Cell preparations and moDC differentiation - Day 0-7

1w 0d 3h

- 1 Make PBMCs using preferred method from whole blood

- 2 Take whole PBMCs and enrich for CD14⁺ cells using magnetic activated cell sorting (MACS) with CD14⁺ microbeads
- 3 Count monocytes, then seed in a 96 well plate at 150,000 cells/well in 200 ul RPMI + 10% P/S + 5% human AB serum (HAB) + 50 ng/ml rhIL-4 and 50 ng/ml rhGM-CSF (day 0)

Incubate in 37°C incubator for 7 days refreshing half media on day 3 and 6
 - 3.1 Each media refresh should be done with double concentration IL-4 and GM-CSF to account for half media change: remove 100 ul media, add 100 ul RPMI + 10% P/S + 5% HAB + 100 ng/ml rhIL-4 and 100 ng/ml rhGM-CSF

Day 6 media change, plus 100 ng/ml LPS for activation : remove 100 ul media, add 100 ul RPMI + 10% P/S + 5% HAB + 100 ng/ml rhIL-4 and 100 ng/ml rhGM-CSF + 200 ng/ml LPS
 - 3.2 **Optional:** collect monocytes (day 0), immature moDCs (day 6) and mature moDCs (day 7) to check purity of MACS enrichment by flow cytometry

Monocytes: CD14⁺
Immature DCs: CD14⁻, CD80⁺, CD86⁺, HLA-ABC⁺, HLA-DR⁺
Mature DCs: CD14⁻, CD80⁺, CD86⁺, HLA-ABC⁺, HLA-DR⁺, CD83⁺
- 4 Using the CD14⁻ population, enrich for T cells using MACS with a PanT negative selection kit
 - 4.1 If specific T cell population is required, perform MACS or FACS (fluorescence activated cell sorting) for required population
 - 4.2 **Optional:** collect PanT to check purity of MACS enrichment by flow cytometry
- 5 Stain T cells with proliferation dye as per preferred protocol

- 6 Count then freeze the stained-T cells in FBS + 10% DMSO for the duration of the monocyte-DC differentiation
- 7 Prepare your "cells of interest" (COI) ready to have enough for 150,000 cells per well on day 7

Seeding the immunogenicity assay - Day 7 (assay Day 0)

2h

- 8 Thaw proliferation dyed-T cells as per preferred protocol
- 9 Remove all media from moDCs and wash once with PBS (moDCs are adherent but aspirate gently to avoid disturbing cells)
- 10 **Experimental wells:**
Seed 150,000 autologous T cells per well in 150 ul RPMI + 10% P/S + 5% HAB
Seed 150,000 COI per well with 150 ul media specific to cells of interest (+ ROCKi if required)

Final: 1:1:1 ratio of moDC:T cell:COI in 300 ul of 50:50 media RPMI media: COI media
- 10.1 **Note:** optimisation will be required to ensure both cell types survive and proliferate as expected in the 1:1 media mix
- 11 **Positive control wells:**

(1) Allogeneic Tcell-moDC
Seed 150,000 allogeneic T cells into moDC wells in 150 ul RPMI + 10% P/S + 5% HAB
Add 150 ul media specific to cells of interest

(2) Polyclonally activated T cells
Seed 150,000 T cells into an empty well (no moDC) in 150 ul RPMI + 10% P/S + 5% HAB
Add activation beads at recommended ratio in 150 ul media specific to cells of interest

Immunogenicity assay - Day 7-12 (assay Day 0-5)

5d

12 Incubate co-culture for 5 days, refreshing half media every 48 hours (day 2 and day 4)

Each change: remove 150 ul media and add 150 ul prepared 1:1 media

Readouts - Day 12 (assay Day 5)

2d

13 For cytokine assays:

Collect 150 ul supernatant per well into a v-bottom 96 well plate

13.1 Centrifuge supernatants plate 1500 g x 10 minutes then re-collect supernatants avoiding any pellet/debris that have collected

13.2 Freeze supernatants at -80°C until use.

Perform ELISA or luminex cytokine assays including IFN γ and TNF α as readouts of immunogenicity.

14 For flow cytometry:

Collect remaining supernatants containing non-adherent T cells by agitating gently, washing the well 1-2 times with the supernatant.

Note: moDCs are adherent so will not be collected, consider that COI may be adherent or non-adherent (it is ok if some are collected as they will be gated out during flow cytometry).

14.1 Perform flow cytometry immediately, including a minimum panel of: CD3, CD4 and CD8 (T cell markers), CD25 (activated T cell marker) and live/dead stain.

Note: cells are already pre-stained with proliferation dye

Activated T cells (CD25+, proliferation dye low/negative) are used as readout of immunogenicity of COI