VERSION 1

FEB 27, 2023

OPEN ACCESS

Protocol Citation: Annabel J Curle, Sarah Howlett, Joanne Jones 2023. MoDC immunogenicity assay. **protocols.io** https://protocols.io/view/modc-immunogenicity-assay-cp3vvqn6

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

Created: Feb 27, 2023

Last Modified: Feb 27, 2023

PROTOCOL integer ID: 77653

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

MoDC immunogenicity assay V.1

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ABSTRACT

The monocyte-derived dendritic cell (moDC) immunogenicity assay can be utilised to test immunogenicity of different cell types. Similar to the mixed lymphocyte reaction (MLR) assay, cells of interest are co-cultured with T cells. Professional antigen presenting cells (APCs; moDCs) are added here to ensure optimal T cell activation in the case of immunogenic cells and improve limited sensitivity of the MLR-like assays.

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 IFNg/TNFa 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-well variation is minimal, but samples can be run in technical duplicates if desired.

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^{o}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)

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^{O}C until use.

Perform ELISA or luminex cytokine assays including IFNg and TNFa 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