Ex vivo stimulation of peripheral blood mononuclear cells (PBMC)

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1 Works for me

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ABSTRACT

Stimulation of PBMCs to study TH1 and TH17 Lymphocytes.

PBMCs were incubated in medium alone, or stimulated for 5 hours with functional mAbs specific for CD3 and CD28, 0.05 µg/ml lipopolysaccharide (LPS), or 50 ng/ml phorbol myristate acetate (PMA) and 1 µg/ml ionomycin.

Wells intended for intracellular staining for IL-23, IL-12, IL-17A, and IFN-γ were stimulated in the presence of brefeldin A to prevent cytokine release from the cells.

Antibody panels with 0.2% saponin were used for intracellular staining of IL12, IL23, IL17A and IFNγ.

Antibody panels with Permeabilization Buffer (Miltenyi FoxP3 Staining Buffer Set) were made for intracellular staining of RORγ(t) and T-Bet.

EXTERNAL LINK

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THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Coakley JD, Breen EP, Moreno-Olivera A, Al-Harbi AI, Melo AM, O'Connell B, McManus R, Doherty DG, Ryan T (2019) Dysregulated T helper type 1 (Th1) and Th17 responses in elderly hospitalised patients with infection and sepsis. PLoS ONE 14(10): e0224276. doi: 10.1371/journal.pone.0224276

MATERIALS TEXT

P/I = phorbol myristate acetate/ionomycin

LPS= lipopolysaccharide

RPMI= RPMI 1640 containing GlutaMAX, 10% HyClone Foetal Bovine Serum, 50 mg/mL streptomycin, 50 U/mL penicillin, 2.5 µg/ml amphotericin B - fungizone, and 25 mM HEPES

PBA= phosphate buffered saline or PBS containing 1.5% bovine serum albumin and 0.02% sodium azide

PBS= phosphate buffered saline

PFA= Paraformaldehyde

FoxP3 Staining Buffer Set

Fluorochrome-conjugated monoclonal antibodies:
CD3 (OKT3), CD28 (15E8), Thermofisher LIVE/Dead Fixable Near IR Dead Cell Stain, IL-12 (REA121), IL-23 (727753), IL-17A (CZ8-23G1), IFN-γ (REA600),. RORγt (REA278), T-bet (REA102), CD 14 (REA599, TÜK4), HLA-DR (REA805), CD16 (REA423), CD19 (LT19), CD3 (REA613), CD4 (REA623), Vδ1 (REA173), CD8 (REA734), Vδ2 (REA771, 123R3), CD161 (191B8), CD56 (REA196), Vα7.2 (REA179), CD27 (REA499), CD45RA (REA562), CD161 (REA631, 191B8), and CD197(REA546). (purchased from Miltenyi Biotec, Gladbach Bergische,

Germany and R&D Systems, Abingdon, UK).

Optimal quantities to give full staining with minimal amount of each antibody were determnined for each vial to ensure optimal results prior to calculation of volume needed.

BD FACS CANTO II flow cytometer

1 Label a 96 well flat bottomed microtiter plate:

A = Medium (no stimulation)
B = P/I
C = LPS
D= CD3/CD28 coated wells

Medium	P/I	LPS	No CD3/CD28
Medium	P/I	No LPS	CD3/CD28 bound
Medium	P/I	No LPS	CD3/CD28 bound
Medium	P/I	No LPS	CD3/CD28 bound
Medium	P/I	No LPS	CD3/CD28 bound
Medium	P/I	No LPS	CD3/CD28 bound
Medium	P/I	No LPS	CD3/CD28 bound

2 Coat CD3/CD28 wells (D2-D7):

Binding 1 µg/ml of each mAb to the wells in 0.1 M Na_2HPO_4, adjusted to pH 9 with 0.1 M NaH_2PO_4, for 4 to 24 hours. Once complete, aspirate wellsand wash once with 100mcl PBS

3 Transfer $0.5x10^6$ PBMCs to the wells in 200 mcl complete RPMI medium

4 Add 50 ng/ml PMA and 1 mcg/ml Ionomycin to wells B1-B7
Add 0.05 mcg/ml LPS to well C1
Add 10 mcg/ml Brefeldin A to wells A1-A3 and A6, B1-B3 and B6, C1, D2, D3, and D6.

5 Incubate cells for 5 hours at 37^oC, 5% CO_2
Prepare antibody cocktails made up to 50mcl PBA and label canto tubes

6 Prepare antibody panels for cell surface staining and made up to 50mcls PBA per sample.

Prepare antibody panels were for intracellular staining and make up to 50mcl with 0.2% saponin (in PBA buffer) for IL12, IL23, IL17A and IFNγ per sample.

Antibody panels for intracellular staining of RORγ(t) and T-Bet are made up to 50mcl with Permeabilization Buffer (Miltenyi FoxP3 Staining Buffer Set)

7 Transfer PBMCs to flow cytometry tubes after mixing well in wells and avoiding bubbles.
Add 1ml PBS.
Centrifuge at 1500RPM for 5 minutes.
Resuspend in dead cell stain and vortex. Incubate for 15 minutes at room temperature in dark.

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8 Add 1ml PBA, vortex, and centrifuge as per step 7.

9 Add antibody cocktail, vortex, incubate for 15 minutes in the dark.
Then add 1ml PBA, vortex, and then centrifuge as per step 7.

10 Fix cells and permeabilise for intracellular staining as follows

11 For wells 1,2,3, and 6 for A,B,C, and D:

Fix with 0.5mls 4% PFA per tube and incubate for 10 minutes in the dark.
Add 1ml PBA, vortex, and then centrifuge as per step 7.
Permeabilise with 1ml 0.2% Saponin in dark for 10 minutes.
Centrifuge as per step 7.
Add intracellular antibody cocktails, vortex, and incubate for 20 minutes in the dark.
Add 1ml PBA, vortex, and centrifuge as per step 7.
Resuspend in 1% PFA 0.5mls for 10 minutes in the dark.
Add 1ml PBA, vortex, and centrifuge as per step 7.
Top up with few drops of PBA.
Store in fridge while acquiring with flow cytometer.

12 For wells 4,5,and 7 for A,B,C, and D:

Add 1ml Fixation.Permeabilisation Solution.
Incubate for 30 minutes in the dark.
Add 1ml PBA, vortex, and centrifuge as per step 7.
Add 1ml Permeabilisation Buffer, vortex, and centrifuge as per step 7.
Add intracellular antibody cocktail, vortex, and incubate for 30 minutes in the dark in the fridge.
Add 1ml cold permeabilisation buffer and centrifuge as per step 7.
Resuspend in 1% PFA, vortex, and incubate in the dark for 10 minutes.
Add 1ml PBA, vortex, and centrifuge as per step 7.
Top up with few drops of PBA.
Store in fridge while acquiring with flow cyometer.