Standard protocols for detection of human CMV-specific T cells

Paloma Riquelme (paloma.riquelme@ukr.de)
Department of Surgery, University Hospital Regensburg, Germany https://orcid.org/0000-0001-8962-3156

James A. Hutchinson
Department of Surgery, University Hospital Regensburg, Germany https://orcid.org/0000-0002-1199-4210

Method Article

Keywords: Flow Cytometry, Cytomegalovirus, CMV, intracellular cytokine staining, IFN-γ, TNF-α, IL-17, IL-4, CD69

DOI: https://doi.org/10.21203/rs.3.pex-758/v1

Abstract

This document describes protocols used for *in vitro* restimulation of cryopreserved PBMC with CMV lysates and subsequent detection of CMV-specific $CD4^+$ and $CD8^+$ T cells by intracellular staining of cytokines.

Introduction

This protocol describes detection CMV-reactive T cells in human PBMC. Briefly, PBMC are stimulated with CMV lysate for 18 hours. Afterwards, CMV-reactive $CD4^+$ and $CD8^+$ T cells are detected by intracellular staining of cytokines, including IFN-γ. Our group has applied this method to frozen PBMC samples from patients with advanced melanoma.

Reagents

Reagents

RPMI1640 without phenol red (BE12918F, Lonza-Biozym)

FCS (10270, Gibco)

DMSO (D2650, Sigma)

Glutamax100X (35050038, Gibco-life)

Pen-Strep 100X (15140-122, Gibco-life)

Human AB Serum, heat-inactivated pooled male-only (ZKT Tübingen)

CMV Lysate (AR03041PU-N, Origene)

αCD28 (340975, BD)

αCD49d (340976, BD)

αPD-1 (MAB10864, R&D)

αCTLA-4 (MAB3254, R&D)

Mouse IgG1 Isotype Control (MAB002, R&D)

Protein Transport Inhibitor Cocktail 500X (00-4980-93, eBioscience)

Cell Stimulation Cocktail 500X (00-4970-93, eBioscience)

SEB (S4881-1MG, Sigma-Aldrich)

DPBS without Ca2+ or Mg2+ (D8537, Sigma)

Cell Staining Buffer (420201, BioLegend)

FcR Blocking Reagent (120-003-855, Miltenyi Biotec)

IC Fixation Buffer (00-8222, eBioscience)

Permeabilization Buffer (00-8333, eBioscience)

Ecotainer Aqua dest. (0082423E, Braun)

ViaKrome 808 Fixable Viability Dye (C36628, Beckman Coulter)

Human FcR blocking reagent (130-059-901, Miltenyi)

Brilliant Stain Buffer Plus (566385, BD)

Antibodies: see Figure 1

Materials

Cryotubes (72.380, Sarstedt)

Freezing Container, “Mr. Frosty” (5100-0001, Nalgene)

96-well plate round bottom (3399, Corning)

96-well plate polypropylene, V-bottom (3357, Corning)

Equipment

Cytoflex LX cytometer: U3-V5-B3-Y5-R3-I2 (Beckman Coulter)

CytExpert version 2.4 Acquisition and Analysis Software (Beckman Coulter)

Kaluza analysis software v.2.1 (Beckman Coulter)

Procedure

1. REAGENT PREPARATION

1.1 Freezing Medium 1

1. Add 20 ml FCS to 30 ml RPMI 1640
2. Freezing medium 1 can be stored up to 1 month at 4°C

1.2 Freezing Medium 2

1. Add 10 ml DMSO to 40 ml FCS
2. Freezing medium 2 can be stored up to 1 month at 4°C

1.3 Culture medium

1. Add 9.3 ml of RPMI 1640 into a falcon tube.
2. Add 0.5 ml of human AB serum (HABS)
3. Add 0.1 ml of GlutaMax
4. Add 0.1 ml of Pen-Strep

2. CELL FREEZING

1. Pre-cool cryotubes, freezing container and all reagents to 4°C,
2. Work on ice using cold reagents in the laminar flow hood
3. Add 1 ml Freezing Medium 1 to up to 20 x 10^6 pelleted PBMC
4. Incubate 20 min on ice
5. Slowly add 1 ml cold Freezing Medium 2
6. Mix by gently pipetting
7. Transfer 1 ml of cell suspension (10 x 10^6 cells/ml) per cryotube
8. Place cryotubes in a freezing container and store at -80°C
9. After 1 day, remove cryotubes from freezing container and store them at -80°C for up to 6 months

10. Alternatively, samples can be stored in liquid nitrogen for longer than 6 months

3. CELL THAWING

1. Pre-warm water bath to 37°C
2. Thaw cells quickly by swirling the cryotube in the water bath (<60 seconds)
3. Before last ice crystal melts, place cryotube in a cold rack
4. Transfer the content of the cryotube into a falcon tube
5. Add 10 ml of cold PBS containing 10% human AB serum dropwise
6. Count cells
7. Centrifuge at 200 g for 10 min
8. Aspirate supernatant using vacuum pump
9. Resuspend cell pellet in culture medium at up to 10 x 10^6/ml
10. Add 150 µl of cell suspension per well in a 96-well plate
11. Incubate for 6 hours at 37°C 5% CO_2 before proceeding with stimulation

4. IN VITRO RESTIMULATION WITH CMV LYSATE

1. Add 5 µg/ml CMV lysate to each well.
2. Add 1µg/ml αCD28 and αCD49d to each well.
3. As a negative control, leave one unstimulated well
4. Optionally, add 5µg/ml αPD1, αCTLA-4 or isotype control to the corresponding wells
5. Incubate for 18 hours at 37°C 5% CO_2
6. In the last 4 hours of incubation, add 2 µl/ml Protein Transport Inhibitor Cocktail to all the wells (0.3 µl 500X stock per well containing 150 µl medium)

7. As a positive control, add 10 µg/ml SEB for 18 hours or alternatively 2 µl/ml Cell Stimulation Cocktail to one well for the last 4 hours.

5. INTRACELLULAR CYTOKINE STAINING

1. Transfer cells from culture wells into a polypropylene V bottom 96 well plate
2. Centrifuge at 1400rpm for 3 min
3. Resuspend pellet in 20 µl PBS containing ViaKrome Fixable Viability Dye 808 (32,5µl dye/ml)
4. Incubate for 20 min at RT in the dark
5. Wash samples with 180 µl Cell Staining Buffer (CSB) by centrifugation at 1400 rpm for 3 min
6. Aspirate supernatant using vacuum pump
7. Add 10 µl 10% FcR block in CSB and shake at 800 rpm for 60" in a rotary shake
8. Incubate at 4 °C for 15 min in the dark
9. Add 20 µl surface antibodies mastermix as indicated in Figure 1
10. Shake at 800 rpm for 5 min and incubate for further 25 min at 4°C in the dark
11. Wash samples with 170 µl CSB by centrifugation at 1400 rpm for 3 min
12. Aspirate supernatant using vacuum pump
13. Add 40 µl CSB to each well and resuspend cell pellets
14. Add 50 µl IC Fixation Buffer
15. Incubate for 30 min at RT in the dark
16. Wash samples with 110 µl Perm Buffer by centrifugation at 1400 rpm for 3 min
17. Aspirate supernatant using vacuum pump
18. Add 40 µl intracellular antibody mastermix as indicated in Figure 1.
19. Shake at 800 rpm for 5 min and incubate for further 25 min at RT in the dark
20. Wash samples with 160 µl Perm Buffer by centrifugation at 1400 rpm for 3 min

21. Aspirate supernatant using vacuum pump
22. Wash samples with 200 µl Perm Buffer by centrifugation at 1400 rpm for 3 min
23. Aspirate supernatant using vacuum pump
24. Resuspend in 150 µl CSB for analysis by flow cytometry

6. ANALYSIS OF DATA

Our gating strategy for analysis of IFN-γ-producing T cells is shown in Figure 2. Samples were acquired using a Cytoflex LX cytometer (Beckman Coulter) and analysed with Kaluza analysis software v.2.1

Troubleshooting

Time Taken

Anticipated Results

References

Acknowledgements