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Human Treg cell suppressive assays

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Abstract

This protocol describes step by step how to perform a suppressive assay with human Treg cells recovered from blood sample.

Subject terms: Cell biology Cell culture Immunological techniques Isolation, Purification and Separation

Keywords: Treg human purification suppressive assay TNF

Introduction

In vitro Treg suppression assays are performed to determine the suppressive function of Treg cells on Teff cells. They are performed by co-culturing the responding population (Teff cells) with the Treg cells stimulated by APC or anti-CD3 coated beads.

Reagents

- Blood sample
- anti-CD25 coated CliniMACS (Miltenyi Biotec)
- anti-CD4 coated (Miltenyi Biotec)
- anti-CD14 coated (Miltenyi Biotec)
- anti-CD19 coated (Miltenyi Biotec)
- LS columns (Miltenyi Biotec)
- Baxter medium (PBS 1X supplemented with 5% of sodium citrate and 5% of human serum albumine)
- Culture medium (RPMI-1650 medium (Gibco) supplemented with 10% heat-inactivated FCS (Gibco or Hyclone), 292 µg/ml or 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco))
- IL-2 (Novartis)
- TNF-α (PeproTech and R&D Systems)

- U-bottom 96-well plates (Corning Costar, 3799)
- 48-well plates (Corning Costar, 3548)
- CFSE (Sigma-Aldrich)
- anti-CD3/CD28 beads (LifeTechnologies)

“List of antibodies”:http://www.nature.com/protocolexchange/system/uploads/3807/original/Table1.?1438096118

Equipment

MidiMACS separator
MACS Multistand
FACS Aria (BD biosciences)
FACS LSRII (BD biosciences)

Procedure

PBMC isolation by Ficoll Hypaque gradient centrifugation
1. recover the blood sample
2. put 15 ml of Ficoll Hypaque in 50 ml falcon and carefully add 30 ml of blood
3. repeat it for the whole blood sample
5. centrifugate at 400G during 22 minutes without break at RT
6. recover and pool the rings containing the PBMC in Baxter medium in 50 ml falcon
7. wash and centrifugate at 200G during 13 minutes at 4°C to separate platelet
8. recover the PBMC pellet in Baxter medium and count it at 1/100

Magnetic separation of Treg cells
1. centrifugate the PBMC at 300G during 5 minutes at 4°C
2. incubate the PBMC with the anti-CD25 beads : 4.5×10^8 cells per ml were incubated with 50 µl of beads for 20 min at 4°C in Baxter medium
3. wash and centrifugate at 300G during 5 minutes at 4°C
4. resuspend at 2×10^8 cells per ml in Baxter medium
5. perform the MACS separation using LS column according to Miltenyi protocol
6. recover the negative fraction (CD25- containing the Teff cells and APC) in a 50 ml falcon and the positive fraction (CD25+ containing the Treg cells) in a 5 ml falcon
7. Count both fraction
Note : the protocol can be stopped at this step over night

Magnetic separation of Teff cells
1. centrifugate about 100-150M cells from the CD25- fraction at 300G during 5 minutes at 4°C

2. incubate them with the anti-CD4 beads : 1×10^8 cells per ml were incubated with 10 µl of beads for 20 min at 4°C in Baxter medium
3. wash and centrifugate at 300G during 5 minutes at 4°C
4. resuspend at 2×10^8 cells per ml in Baxter medium
5. perform the MACS separation using LS column according to Miltenyi protocol
6. recover the negative fraction (CD25-CD4- containing the APC) in a 50mL falcon and the positive fraction (CD25-CD4+ containing the Teff cells) in a 5mL falcon
7. Count both fraction

Note : Teff cells are frozen if they have to be used another day

Magnetic separation and preparation of APC (if needed)

1. centrifugate the CD25-CD4- fraction at 300G during 5 minutes at 4°C
2. incubate them with the anti-CD14 and anti-CD19 beads : 1×10^8 cells per ml were incubated with 10 µl of each bead type for 20 min at 4°C in Baxter medium
3. wash and centrifugate at 300G during 5 minutes at 4°C
4. resuspend at 2×10^8 cells per ml in Baxter medium
5. perform the MACS separation using LS column according to Miltenyi protocol
6. recover the positive fraction (CD25-CD4-CD14+CD19+, containing the APC) in a 5mL falcon
7. wash in culture medium and centrifugate at 300G during 5 minutes at 4°C
8. irradiate the APC at 50 Grey
9. wash in culture medium and centrifugate at 300G during 5 minutes at 4°C
9. Count the APC fraction

FACS sorting of Treg cells

1. centrifugate the CD25+ fraction at 300G during 5 minutes at 4°C
2. incubate the pellet at 5×10^7 cells per ml during 20 minutes at 4°C with the following antibodies :

anti-CD4 PerCP (1/20)
anti-CD25 PE (1/20)
anti-CD127 BV451 (1/40)
anti-CD45RA PE-Cyanine7 (1/10)

3. wash and centrifugate at 300G during 5 minutes at 4°C
4. resuspend the pellet at 20×10^6 cells per ml, filter the solution and tranfer it in a 5 ml FACS tube
5. Sort the Treg cells using Aria accordingly :

CD4+CD25+IL-7Ra-CD45RA+ for naive Treg cells
CD4+CD25+IL-7Ra-CD45RA- for memory Treg cells

6. after sorting, wash in PBS and centrifugate Treg cells at 400G during 10 minutes at 4°C
7. wash the cells with culture medium and transfer them in eppendorf tube

8. centrifugate at 300G during 5 minutes
9. count both fraction

Note : from this point Treg cells can be used for suppressive assay or preincubation

Treg cell preincubation (if needed)

1. count and resuspend the cells at 1-3×10^5 cells per 300 ul
2. prepare a solution of IL-2 concentrate 5X at 1500 UI/ml (300 UI/ml final)
3. prepare a solution of TNF concentrate 5X at 250 ng/ml (50 ng/ml)
4. distribute 300 ul of Treg cells in a 48-well plates
5. add 100 ul of the IL-2 or/and the TNF to complete to 500 ul
6. incubate at 37°C during 20h to 72h

CFSE labelling of the Teff cells

1. wash the Teff cells in PBS and centrifugate them at 300G during 5 minutes at 4°C
2. repeat the step 1.
3. incubate the Teff cells with 1 uM CFSE at 1×10^7 cells per ml in PBS during 5 minutes at RT
4. stop the reaction with 1/5 of FCS

during 1 minute

5. wash in Complete medium and centrifugate the cells at 300G during 5 minutes at 4°C
6. repeat step 5.
7. Count the Teff cells

All suppressive assays are performed in U-bottom 96-well plates with 10^4 CFSE labelled Teff cells and 10^4 to 1.25×10^3 Treg cells (1:1 to 1:8) in 200 µl of culture medium at 37°C 5% CO2 during 4 days. Some wells with Teff cells alone are used as positive control of proliferation and are also used to determine the direct effect of TNF on Teff cells.

Suppressive assay with beads

1. resuspend the Treg cells at 10^4 cells per 50 ul and make serial dilution (1 to 2)
2. resuspend the Teff cells at 10^4 cells per 100 ul
3. prepare a solution of TNF concentrate 4X at 200 ng/ml (50 ng/ml final) if needed
4. add anti-CD3/CD28 beads to the Teff cells solution to be at 10^3 beads per 100 ul
5. distribute the different solutions : Teffs cells + beads (100 ul), Treg cells (50 ul) then TNF (50 ul) in the appropriate wells in duplicate for each condition.

Suppressive assay with APC and coated anti-CD3

1. coat a U-bottom 96-well plate with 0.5 ug/ml of anti-CD3 (100 ul per well) during 2h at 37°C
2. resuspend the Treg cells at 10^4 cells per 50 ul and make serial dilution (1 to 2)
3. resuspend the Teff cells at 10^4 cells per 50 ul

4. resuspend the APC at 10^5 cells per 50 ul and mix it at equal volume with the Teff cells solution
5. prepare a solution of TNF concentrate 4X at 200 ng/ml (50 ng/ml final) if needed
6. Wash the plates 2 times with 150 ul of PBS without letting the plate dry
7. distribute the different solutions : Teffs cells + APC (100 ul), Treg cells (50 ul) then TNF (50 ul) in the appropriate wells in duplicate for each condition.

Suppressive assay with APC and soluble anti-CD3
1. resuspend the Treg cells at 10^4 cells per 50 ul and make serial dilution (1 to 2)
2. resuspend the Teff cells at 10^4 cells per 50 ul
4. resuspend the APC at 10^5 cells per 50 ul and mix it at equal volume with the Teff cells solution
5. add anti-CD3 to the Teff cells + APC solution to be concentrate 2X at 10 ug/ml (5 ug/ml final)
6. prepare a solution of TNF concentrate 4X at 200 ng/ml (50 ng/ml final) if needed
7. distribute the different solutions : Teffs cells + APC + anti-CD3 (100 ul), Treg cells (50 ul) then TNF (50 ul) in the appropriate wells in duplicate for each condition.

FACS Analysis
1. centrifugate the U-bottom 96-well plate at 300G during 5 minutes at 4°C
2. incubate the cells in 50 ul of PBS+3%FCS during 15 minutes at 4°C with the anti-CD4 PerCP antibodies Biolegend (1/100)
3. wash in PBS+3%FCS and centrifugate the plate at 300G during 5 minutes at 4°C
4. resuspend in 200 ul of PBS+3%FCS and analyse by FACS

Calcul of the percentage of suppression
For each Teff/Treg ratio, the percentage of suppression was calculated with the following formula: [Log2(y) of (Teff cells alone) – Log2(y) of (Teff + Treg cells]) / Log2(y) of (Teff cells alone) X 100. The y value corresponds to the mean fluorescent intensity of CFSE of the whole Teff cell population divided by the mean fluorescent intensity of CFSE of undivided Teff cells.

Figures

Table1: List of antibodies

Download Table1
List of antibodies

Author information

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Competing financial interests

The authors declare no competing financial interests.

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Readers' Comments

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Protocol Exchange ISSN 2043-0116