A protocol for the role of TNF-α on regulatory T cell function

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Jingwu Zhang
BioSciKin, Nanjing, China.

jwzang@yahoo.com*Corresponding Author*

Hong Nie
Shanghai Institute of Immunology, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

Yingxia Zheng
Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China.

Runsheng Li
Shanghai Qianchen Biotechnologies, Ltd., Shanghai, China.

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Abstract

In vitro Treg suppression assays are performed to determine the functional effect of TNF-α on Treg cells. In addition to the co-culture condition to measure the effect of TNF-α on human Treg by mixing them with Teff cells, parallel experiments were conducted where TNF-α was washed away prior to co-culture (pre-treatment condition).

Reagents

-Recombinant human TNF-α (R&D Systems, Cat: 210-TA-020), rhIL-2 (Roche, Cat: 11011456001), CFSE (Invitrogen, Cat: C34554), anti-CD3/CD28 beads (Life Technologies, Cat: 111.31D), Anti-Human CD4 PerCP-Cyanine5.5 (eBioscience, Cat: 45-0048), Anti-Human CD25 APC (eBioscience, Cat: 17-0259), Anti-Human CD127 PE (eBioscience, Cat: 12-1278), Human $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec, Cat: 130-096-533), Lymphoprep (Axis-shield, Cat: 1114546)

-Culture medium: RPMI-1640 (Gibco) supplemented with 10% heat-inactivated FBS (Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco).

-MACS Buffer: 1×PBS supplemented with 0.5% BSA and 2 mM EDTA.

-FACS Buffer: 1×PBS supplemented with 2% FBS.

Equipment

FACSAria II (BD Biosciences) for sorting

FACSCanto II (BD Biosciences) for analysis

FACS tubes round bottom (BD Biosciences)

96-well U-bottom plates (Corning Costar)

LS columns (Miltenyi Biotec)

CO_2 incubator (Thermo)

Procedure

Isolation of Treg and Teff cells

1. Obtain fresh blood specimens from healthy donors and isolate PBMC by Lymphoprep density gradient medium.
2. Isolate total $CD4^+$ T cells using the $CD4^+$ T cell Isolation Kit as the following steps in

brief. (1) Resuspend total PBMC in 40 µl of MACS buffer per 10^7 cells, and incubate with 10 µl of Biotin-Antibody Cocktail per 10^7 cells for 10 min at 4°C.

(2) Subsequently, add 30 µl of MACS buffer per 10^7 cells, and incubate cells with 20 µl of MicroBead Cocktail per 10^7 cells for 15 min at 4°C.

(3) Wash cells with MACS buffer and resuspend cells in 1 ml of MACS buffer.

(4) Prepare LS column by rinsing with 3 ml of MACS buffer.

(5) Apply cell suspension onto the column and collect unlabeled cells that passed through.

3. Wash $CD4^+$ T cells (negative fraction) with MACS buffer.

4. Resuspend $CD4^+$ T cells with 100 µl of FACS buffer, and add 10 µl anti-CD4, anti-CD25, anti-CD127, respectively, mix and incubate for 15 min at 4°C.

5. Wash cells twice with 3 ml of FACS buffer, follow by FACS sorting for $CD4^+CD25^{hi}CD127^{low/-}$ as Treg cells and $CD4^+CD25^-$ T cells as Teff cells. The purity of Treg cells and Teff cells ranged 95%-98%.

Assay setup

6. Treat Treg cells with TNF-α (50 ng/ml) or leave them in medium alone (control) in 96-well U-bottom plates ($0.5\text{-}1.0\times10^5$ cells per well) in the presence of IL-2 (100 U/ml) for 24 hs.

7. Wash the resulting Treg cells twice with culture medium, and count the cells.

8. Label Teff cells with CFSE at 2 µM, 37°C for 8 min, then wash for three times with culture medium.

9. Inhibition assay in different conditions as follows. (1) TNF-α pre-treatment assay: culture CFSE labeled Teff cells (1×10^4 cells per well) alone or with Treg cells (1×10^4 cells per well) that were either untreated or pretreated with TNF-α (50 ng/ml) in 96-well U-bottom plates. Maintain the culture in the presence of 2×10^3 anti-CD3/CD28

beads for 4 days.

(2) TNF-α co-culture assay: culture CFSE labeled Teff cells (1×10^4 cells per well) alone or with Treg cells (1×10^4 cells per well) which were not pre-exposed to TNF-α, in the presence or absence of TNF-α (50 ng/ml) in 96-well U-bottom plates. Maintain the culture in the presence of 2×10^3 anti-CD3/CD28 beads for 4 days.

10. Measure proliferation of Teff cells by CFSE dilution in flow cytometry analyses. Express the results as percent inhibition: (1 − (experimental CFSE dilution/control CFSE dilution)) × 100%.