

Adoptive Transfer of Tumor Expanded Regulatory T Cells (Tregs)

Felipe Vences-Catalán* and Shoshana Levy*

Department of Medicine, Division of Oncology, Stanford University, Stanford, CA, USA

*For correspondence: fvences5@stanford.edu; slevy@stanford.edu

[Abstract] Regulatory T cells (Tregs), a subset of CD4⁺CD25⁺ T cells, infiltrate tumors and suppress antitumor activity of effector T and NK cells. Depletion of Tregs by anti CD25⁺ antibodies has been shown to reduce tumor growth and metastasis (Oikhanud *et al.*, 2009). Conversely, adoptive transfer of Tregs induced immune suppression and promoted tumor growth (Smyth *et al.*, 2006; Janakiram *et al.*, 2015). We have adoptively transferred Tregs to evaluate their immunosuppressive function *in vivo*. Our study (Vences-Catalan *et al.*, 2015) compared the immunosuppressive efficacy of Tregs derived from tumor-bearing wild type to those of CD81KO mice. The following protocol could be adapted to any other source of Tregs.

Lymph node or splenic tumor-induced Tregs are isolated and purified by a two-step procedure using CD4⁺CD25⁺ regulatory T cell isolation kit from MACS Miltenyi Biotec. First, CD4⁺ T cells are enriched by negative selection, followed by positive selection of CD25⁺ T cells. Tumor-induced purified Tregs (CD3⁺CD4⁺CD25⁺FoxP3⁺) are then co-injected subcutaneously together with tumor cells into naïve mice (Winn assay) (Winn, 1960). Tregs could also be injected intravenously once or several times, according to the research needs. The effect of the adoptively transferred Tregs on tumor growth is then measured by caliper or by *in vivo* imaging techniques.

Materials and Reagents

1. 70 µm cell strainer (Corning, Falcon®, catalog number: 352350)
2. 1 ml syringe (BD, catalog number: 309659)
3. 30 G x 1/2 needle (BD, catalog number: 305106)
4. LD columns (Miltenyi Biotec, catalog number: 130-042-901)
5. MS columns (Miltenyi Biotec, catalog number: 130-042-201)
6. 15 and 50 ml polypropylene conical tubes (Corning, Falcon®, catalog number: 352096 and 352070)
7. Mice
Note: We use 6-8 weeks old female Balb/c or C57BL6 mice, but the protocol is applicable to any mouse strain as long as both donor and recipient mice are from the same genetic background.
8. Cells

Note: We use breast cancer cell lines 4T1 or E0771 syngeneic to Balb/c or C57Bl6 respectively.

9. Fetal calf serum (FCS) (GE life sciences, HyClone™, FetalClone®III, catalog number: SH30109.03)
10. Penicillin-Streptomycin (Pen-Strep) (Thermo Fisher Scientific, Gibco™, catalog number: 15140-22)

Note: 5 ml of this solution was used in 500 ml of RPMI 1640 media.
11. PBS (Corning, Cellgro, catalog number: 21-031-CV)
12. ACK buffer (Quality Biological, catalog number: 118-156-101)
13. CD4⁺CD25⁺ Regulatory T cell Isolation kit (Miltenyi Biotec, catalog number: 130-091-041)

Components of the kit:

 - a. 1 ml CD4⁺CD25⁺ Regulatory T cell Biotin-antibody cocktail
 - b. 2 ml Anti-biotin Microbeads
 - c. 1 ml CD25-PE
 - d. 1 ml Anti-PE Microbeads
14. RPMI 1640 (Corning, Cellgro, catalog number: 10-040-CV)
15. Bovine serum albumin (BSA) (Sigma Aldrich, catalog number: A9647-500G)

Note: A solution of 0.5% in PBS was used.
16. EDTA (Sigma Aldrich, catalog number: E-5134)

Note: A solution of 2 mM in PBS was used.
17. Optional: CD3 FITC (clone:145-2C11) (BD Pharmingen)
18. Optional: CD25 PE (clone:PC61.5) (BD Pharmingen)
19. Optional: CD4 PerCP (clone:H129.19/RM4-5) (BD Pharmingen)
20. Optional: FoxP3 APC (clone:FJK16s) (BD Pharmingen)
21. MACS Buffer (see Recipes)

Equipment

1. MidiMACS separator (Miltenyi Biotec, catalog number: 130-042-302)
2. MiniMACS separator (Miltenyi Biotec, catalog number: 130-042-102)
3. Centrifuge (Eppendorf, model: 5810 R)

Procedure

A. Induction of regulatory T cells

4T1 or E0771 cells were grown in RPM media, supplemented with 10% FCS and 1% Pen-Strep. Inject subcutaneously $1-5 \times 10^4$ 4T1 or $0.5-1 \times 10^6$ E0771 cells resuspended in PBS (90% or greater viability) into either Balb/c or C57BL6 mice, respectively (an average of 3-5 mice are needed to obtain around $1-5 \times 10^6$ Tregs, if more Tregs are needed inject more mice).

B. Sample preparation for Treg purification

After 12-16 days, euthanize the mice by cervical dislocation (AVMA Guidelines for the Euthanasia of Animals: 2013 Edition) and remove lymph nodes (inguinal and axillary) (Mathieu *et al.*, 2012) and/or spleens. Prepare single-cell suspension by mechanical disruption of the tumor (make sure to work with single cell suspension, by passing through a 70 μm cell strainer).

Optional: If red blood cells are present, lyse with ACK buffer, although it is not necessary as the kit contains anti Ter-119 antibody to positively deplete red blood cells.

Note: Work fast, keep cells cold and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and will reduce non-specific cell labeling.

C. Magnetic labeling of non-CD4⁺ cells and fluorescent labeling of CD25⁺ cells

We follow exactly the protocol provided by the manufacturer to purify CD4⁺CD25⁺ Regulatory T cells. Volumes for magnetic labeling given below are for starting cell number of 10⁷ leukocytes. When working with fewer than 10⁷ leukocytes use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly.

1. Determine the number of leukocytes.
2. Centrifuge cells at 300 x g for 10 min at room temperature (RT). Aspirate supernatant completely.
3. Resuspend cell pellet in 40 μl of MACS buffer per 10⁷ total cells.
4. Add 10 μl of Biotin-Antibody cocktail per 10⁷ total cells (this cocktail contains anti-mouse antibodies against: CD8a, CD11b, CD45R, CD49b and Ter-119).
5. Mix well by pipetting up and down, and refrigerate for 10 min (4-8 °C).
6. Add 30 μl of MACS buffer, 20 μl of anti-biotin Microbeads and 10 μl of CD25-PE antibody per 10⁷ total cells.
7. Mix well and refrigerate for an additional 15 min in the dark (4-8 °C).
8. Wash cells by adding 1-2 ml of buffer per 10⁷ total cells and centrifuge at 300 x g for 10 min RT. Aspirate supernatant completely.
9. Resuspend cell pellet in 500 μl of MACS buffer (up to 1.25 x 10⁸ cells can be suspended in this volume according to the manufacturer).
10. Proceed to magnetic separation.



Figure 1. MACS midi system for cell purification

D. Depletion with LD column

1. Place LD column in the magnetic field of a midi MACS separator (Figure 1).
2. Prepare column by rinsing with 2 ml of MACS buffer.
3. Apply cell suspension to the column.
4. Collect unlabeled cells which pass through and wash column 2 times by pipetting 1 ml of MACS buffer. Perform washing steps by adding MACS buffer successively once the column reservoir is empty. Collect total effluent. This is the unlabeled CD4⁺ T cell fraction.
5. Proceed for the enrichment of CD4⁺CD25⁺ T cells.

E. Magnetic labeling of CD25⁺ cells

Volumes for magnetic labeling given below are for up to 10⁷ initial starting cell number. For larger initial cell numbers, scale up accordingly.

1. Centrifuge isolated CD4⁺ T cells at 300 x g for 10 min. Aspirate supernatant completely.
2. Resuspend cell pellet in 90 µl of MACS buffer.
3. Add 10 µl of anti-PE Microbeads.
4. Mix well and refrigerate for 15 min in the dark (4-8 °C).
5. Wash cells by adding 1-2 ml of MACS buffer and centrifuge at 300 x g for 10 min. Aspirate supernatant completely.
6. Resuspend up to 10⁸ cells in 500 µl of MACS buffer.
7. Proceed to magnetic separation.

F. Magnetic separation: Positive selection of CD4⁺CD25⁺ regulatory T cells

1. Place MS column in the magnetic field of a suitable MACS separator.
2. Prepare column by rinsing with 500 µl of MACS buffer.
3. Apply cell suspension onto the column.
4. Let cells pass through and wash column 3 times with 500 µl of MACS buffer. Perform washing steps by adding MACS buffer three times, once the column reservoir is empty.
5. Remove column from the separator and place it on a suitable collection tube.
6. Pipette 1 ml of MACS buffer on to the column. Immediately flush out the magnetically labeled cells (CD4⁺CD25⁺) by firmly pushing the plunger into the column.
7. Optional: To achieve highest purities, repeat magnetic separation with a consecutive column run.

G. Co-injection of Tregs with 4T1 or E0771 tumor cells

1. Determine the cell number of Tregs and tumor cells needed for injection.
2. Inject approximately 100-200 µl of PBS containing a mixture purified Tregs with tumor cells subcutaneously close to the mammary fat pad.
3. Follow tumor growth by caliper measurements.

Representative data

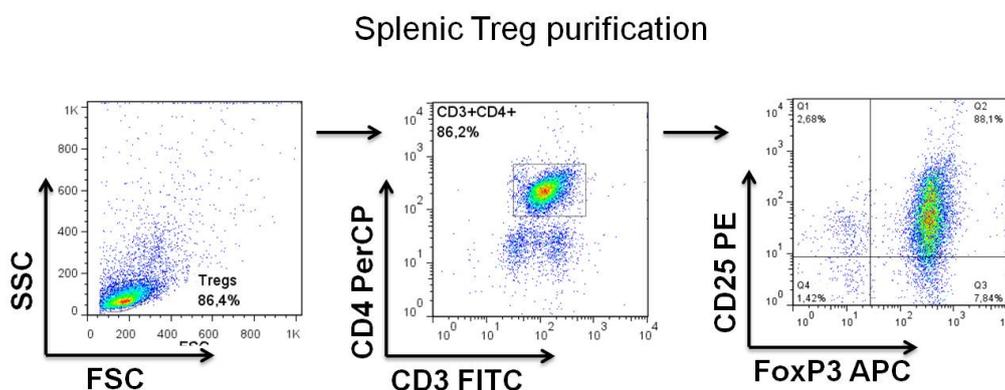


Figure 2. Representative Treg purification

Notes

1. Tregs purity varies from 85-95% (See Figure 2).
2. On our experiments we transferred 5×10^4 4T1 cells together with 1×10^6 Tregs or 0.5×10^6 E0771 cells with 1×10^6 Tregs.

Recipes

1. MACS Buffer
 PBS supplemented with 0.5% BSA and 2 mM EDTA
 Keep buffer cold (4-8 °C)

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