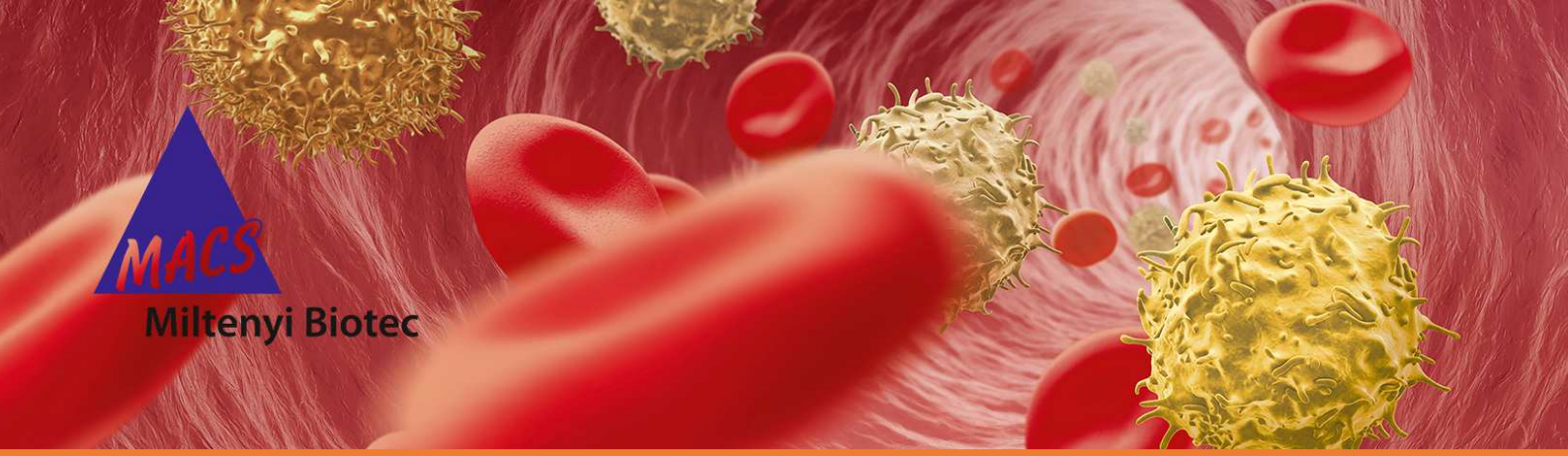

Human CD4$^+$CD25$^+$ CD127$^{dim/-}$ regulatory T cell isolation, *in vitro* expansion and analysis

In vitro human regulatory T cell expansion

Introduction

Regulatory T (Treg) cells are a subpopulation of T cells. Basic as well as clinical research has focused on the study of Treg cells for years due to their unique function of suppressing immune responses. As Treg cells represent a small cell population, many downstream applications require an expansion of Treg cells prior to analysis. However, Treg cells have been described as hypoproliferative in response to polyclonal stimulation and interleukin 2 (IL-2) *in vitro*. Thus, *in vitro* cultivation of Treg cells often results in low expansion rates or low frequencies of FoxP3$^+$ Treg cells due to overgrowth of conventional T cells.

To overcome this problem, we have developed the Treg Expansion Kit, human, which is designed to efficiently expand Treg cells that maintain FoxP3 expression after isolation with the CD4$^+$CD25$^+$CD127$^{dim/-}$ Regulatory T Cell Isolation Kit II, human.

This application note includes a complete protocol for your Treg cell workflow, describing human Treg cell isolation from peripheral blood mononuclear cells (PBMCs), their *in vitro* expansion and subsequent flow cytometry analysis.

Workflow

Day 0	Treg cell isolation Flow cytometry analysis
Day 1	Addition of 100 µL expansion medium
Day 3-5	Split cells or replace 100 µL medium with fresh expansion medium
Day 5-8	Transfer Treg cells into 24-well plate or split cells and use a new 96-well plate
Day 14	Stop Treg expansion and analyze Treg cells by flow cytometry OR Restimulate Treg cells
Day 21	Stop Treg cell expansion Flow cytometry analysis

Materials

Treg cell isolation from PBMCs

Treg cell isolation kit

- $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II, human (# 130-094-775)

Separation buffer

- 1x PBS (pH 7.2), BSA, EDTA
- Alternative: 6× 1.5 L autoMACS® Running Buffer – MACS® Separation Buffer (# 130-091-221)

Magnetic separation

- LD Columns, 1 for 10^8 labeled cells (# 130-042-901)
- MS Columns, 2× per isolation (# 130-042-201)
- MidiMACS™ Separator (Separator for LD Column), (# 130-042-302)
- MiniMACS™ Separator (Separator for MS Column), (# 130-042-102)
- MACS® MultiStand (# 130-042-303)
- (Optional) Pre-Separation Filters, 30 µm (# 130-041-407)

Treg cell *in vitro* expansion

Expansion medium

- TexMACS™ Medium, research grade (500 mL), (# 130-097-196)
- Human IL-2 IS, premium grade (50 µg) (# 130-097-745)
- human AB Serum
- ß-Mercaptoethanol (ß-ME)
- Penicillin/Streptamycin (PenStrep)
- (Optional) Rapamycin

Expansion process

- Treg Expansion Kit, human:
  2 mL for stimulation of 1×10^7 Treg cells (# 130-095-345);
  2×2 mL for stimulation of 2×10^7 Treg cells (# 130-095-353)
- 96-well round-bottom plate
- (Optional) 24-well plate

Bead removal (optional)

- MACSiMAG™ Separator (# 130-092-168)

Staining of Treg cells for flow cytometry analysis

- Treg Detection Kit (CD4/CD25/FoxP3) (PE), human (# 130-094-163)

OR

- Treg Detection Kit (CD4/CD25/FoxP3) (APC), human (# 130-094-158)
- (Optional): CD127- PE-Vio 770 (# 130-109-516) or CD127- PE-Vio 615, human (# 130-107-513)

Material preparation

Treg cell isolation from PBMCs

Separation buffer

- 1x PBS (pH 7.2)
  + 0.5% BSA
  + 2 mM EDTA

Treg cell *in vitro* expansion

Expansion medium

The expansion medium can be stored for up to 10 days under sterile conditions at 4°C.

- 500 mL TexMACS™ Medium
  + 500 IU/mL Human IL-2 IS, premium grade
  + 5% human AB Serum
  + 0.01 mM ß-ME
  + 1% PenStrep
  + (Optional) 100 nmol/L Rapamycin

▲ **Note:** Rapamycin has been shown to increase the frequency of $FoxP3^+$ Treg cell during expansion (Battaglia, M. *et al.* (2005) *Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. Blood 105: 4743–4748).*

Flow cytometry analysis of Treg cell expansion (intracellular staining)

FoxP3 staining buffer

(components provided in Treg Detection Kit)

▲ Always prepare reagents freshly on the same day that intracellular FoxP3 staining is performed.

▲ The required total buffer volumes should be calculated beforehand; volumes will depend on the number of cells to be analyzed as well as the number of tests to be performed.

Example:
For 1 sample with 1×10^6 Treg cells:
prepare 1 mL of fixation/permeabilization solution and 4 mL of 1× Permeabilization Buffer
I) Fixation/permeabilization solution

- Dilute Fixation/Permeabilization Solution 1 (e.g. 0.25 mL) 1:4 with Fixation/Permeabilization Solution 2 (e.g. 0.75 mL).

II) 1× Permeabilization Buffer

- Dilute the 10× Permeabilization Buffer (e.g. 1 mL) 1:10 with deionized or distilled water (e.g. 9 mL).

Methods

1. Generation of PBMCs

When working with anticoagulated peripheral blood or buffy coat, PBMCs should be isolated by density gradient centrifugation, for example, using Ficoll-Paque™ according to the manufacturer's instructions. Collect a sample of PBMCs for subsequent flow cytometry analysis.

2. Isolation of $CD4^+CD25^+CD127^{dim/-}$ Treg cells using the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II, human

2.1 Magnetic labeling of non-$CD4^+$ and $CD127^{high}$ cells
▲ Volumes for magnetic labeling given below are for up to 10^7 total cells. When working with fewer than 10^7 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10^7 total cells, use twice the volume of all indicated reagent volumes and total volumes)

1. Determine cell number.

2. Centrifuge cell suspension at 300×g for 10 minutes. Discard supernatant.

3. Resuspend cell pellet in 40 µL of separation buffer per 10^7 total cells.

4. Add 10 µL of $CD4^+CD25^+CD127^{dim/-}$ T Cell Biotin-Antibody Cocktail II per 10^7 total cells.

5. Mix well and incubate for 5 minutes in the refrigerator (2–8 °C).

6. Add 30 µL of separation buffer and 20 µL of Anti-Biotin MicroBeads per 10^7 total cells.

7. Mix well and incubate for 10 minutes in the refrigerator (2–8 °C).

8. Adjust the volume to a minimum of 500 µL of separation buffer.
▲ **Note:** Resuspend up to 10^8 cells in 500 µL of separation buffer. For higher cell numbers, scale up buffer volume accordingly.

2.2 Magnetic depletion of non-$CD4^+$ and $CD127^{high}$ cells
The first step of Treg cell isolation is a depletion of non-$CD4^+$ and $CD127^{high}$ cells. Here, an LD Column is used, which has a capacity of 10^8 labeled cells and 5×10^8 total cells.
▲ **Note:** When using the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T cell Isolation Kit II, human, we do not recommend to process more than 1.3×10^8 total cells on an LD Column. When exceeding this cell number, it is strongly recommended to split the sample and use additional LD Columns.

1. Place LD Column(s) in the magnetic field of a MidiMACS™ Separator.

2. Prepare column by rinsing with 2 mL of separation buffer. Always wait until the column reservoir is empty before proceeding to the next step.

3. Apply cell suspension onto the column.

4. Collect unlabeled cells that pass through and wash column twice with 1 mL of separation buffer each. **Collect total effluent; this is the unlabeled pre-enriched $CD4^+$ cell fraction which is needed for further Treg cell isolation.**

5. Determine cell number.

2.3. Magnetic labeling of $CD25^+$ cells

▲ Volumes for magnetic labeling given below are for an initial starting cell number of up to 10^7 total cells. For higher initial cell numbers, scale up all volumes accordingly.

1. Centrifuge cell suspension at 300×g for 10 minutes. Discard supernatant.

2. Resuspend cell pellet in 90 µL of separation buffer per 10^7 total cells.

3. Add 10 µL of CD25 MicroBeads II per 10^7 total cells.

4. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).

5. Wash cells by adding 1–2 mL of separation buffer and centrifuge at 300×g for 10 minutes. Discard supernatant.

6. Resuspend up to 10^8 cells in 500 µL of separation buffer.

▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.

2.4. Magnetic separation of $CD25^+$ cells

The second step during Treg cell isolation is a positive selection of $CD25^+$ cells. Here, two consecutive MS Columns are used, with a capacity of 10^7 labeled cells. To not exceed the capacity, it is recommended to determine the frequency of $CD25^+$ cells in your cell suspension by flow cytometry beforehand.

1. Place an MS Column in the magnetic field of a MiniMACS™ Separator.

2. Prepare column by rinsing with 500 µL of separation buffer.

3. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.

4. Wash column 3 times with 500 µL of separation buffer each. Collect unlabeled cells that pass through and combine with the effluent from step 3.

5. Remove column from the separator and place it on a suitable collection tube.

6. Pipette 1 mL of separation buffer onto the column. Immediately flush out magnetically labeled cells by firmly pushing the plunger into the column.

7. To increase purity of $CD4^+CD25^+CD127^{dim/-}$ cells, the eluted fraction can be enriched over a second MS Column (recommended). Repeat the magnetic separation procedure described in steps 1 to 6 using a new MS Column. **The isolated Treg cells are now ready-to-use for *in vitro* expansion.**

To assess the purity and to determine the frequency of FoxP3-expressing cells as start population for expansion, a surface and intracellular flow cytometry staining of the isolated Treg cells must be performed. The purity is determined by surface staining of CD4, CD25 and CD127. Furthermore, for the final analysis of Treg expansion, it is also important to determine:

a) The initial cell number
b) The initial cell number and frequency of FoxP3-expressing $CD4^+$ cells

Please refer to section 3 for detailed protocol.

3. Surface and intracellular flow cytometry staining of Treg cells using the Treg Detection Kit

▲ Volumes given below are **for up to 10^6** nucleated cells. When working with fewer than 10^6 cells, use the same volumes as indicated **except for the fixation step** due to the impact on cell morphology. When working with higher cell numbers, scale up all reagent volumes and total volumes, accordingly (e.g. for 2×10^6 nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

3.1 Surface staining of Treg cells with CD4 and CD25 antibodies (optional: CD127 antibody)

1. Determine cell number.

2. Centrifuge cell suspension at 300×g for 10 minutes. Discard supernatant.

3. Resuspend up to 10^6 nucleated cells per 90 µL of separation buffer.

4. Add 10 µL of CD4-FITC and 20 µL of CD25 antibody (either PE or APC conjugated). (Optional: Add 10 µL of CD127 PE-Vio 770 or CD127-PE-Vio 615)

5. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).

6. Wash cells by adding 1–2 mL of separation buffer per 10^6 cells and centrifuge at 300×g for 5 minutes at 4 °C. Discard supernatant

7. Proceed immediately to 3.2.

3.2 Intracellular staining with the Anti-FoxP3 antibody

1. Resuspend 10^6 nucleated cells in 1 mL cold, freshly prepared fixation/permeabilization solution (for details refer to material preparation section). When working with fewer cells, scale down the buffer volume accordingly (e.g. for $5×10^5$ nucleated cells use 500 µL of fixation/permeabilization solution).

2. Mix well and incubate for 30 minutes in the dark in the refrigerator (2–8 °C).

3. Wash cells by adding 1–2 mL of cold separation buffer per 10^6 cells and centrifuge at 300×g for 5 minutes at 4 °C. Discard supernatant.

4. Wash cells by adding 1–2 mL of cold 1× Permeabilization Buffer (for details refer to material preparation section) per 10^6 cells and centrifuge at 300×g for 5 minutes at 4 °C. Discard supernatant.

5. Resuspend up to 10^6 nucleated cells in 80 µL of cold 1× Permeabilization Buffer.

6. Add 20 µL of FcR Blocking Reagent.

7. Mix well and incubate for 5 minutes in the refrigerator (2–8 °C).

8. Add 10 µL of the Anti-FoxP3 antibody (either APC or PE).

9. Mix well and incubate for 30 minutes in the dark in the refrigerator (2–8 °C).
10. Wash cells by adding 1–2 mL of cold 1× Permeabilization Buffer per 10^6 cells and centrifuge at 300×g for 5 minutes at 4 °C. Discard supernatant.

11. Resuspend cell pellet in a suitable amount of separation buffer for analysis by flow cytometry.
▲ **Note**: Due to fixation and permeabilization, cells can be smaller than viable cells. Thus, FSC/SSC settings of the flow cytometer might need to be adjusted.

3.3. Flow cytometry analysis

To assess the purity of the isolated Treg cells and the initial frequency of $FoxP3^+$ $CD4^+$ cells, the cells were analyzed by flow cytometry. The analysis should be performed with the cell sample taken in section 1 (before separation) and cell sample after isolation (after separation).

1. Lymphocytes were identified by FSC/SSC gating (data not shown).

2. Dead cells were excluded from the analysis by using the live/dead cell exclusion marker PI (data not shown).

3. $CD4^+$ cells were identified according to CD4/FSC analysis among lymphocytes (data not shown).

4. $CD4^+$ cells were further analyzed for their expression of CD25 (y-axis) and absence of CD127 (x-axis) to assess the purity of $CD25^+CD127^{dim/-}$ cells among $CD4^+$ cells (fig. 1 A and B, left plots).

5. Lymphocytes were further analyzed for their expression of CD4 (y-axis) and FoxP3 (x-axis) to assess the initial frequency of FoxP3-expressing $CD4^+$ cells among lymphocytes (fig. 1 A and B, right plots).

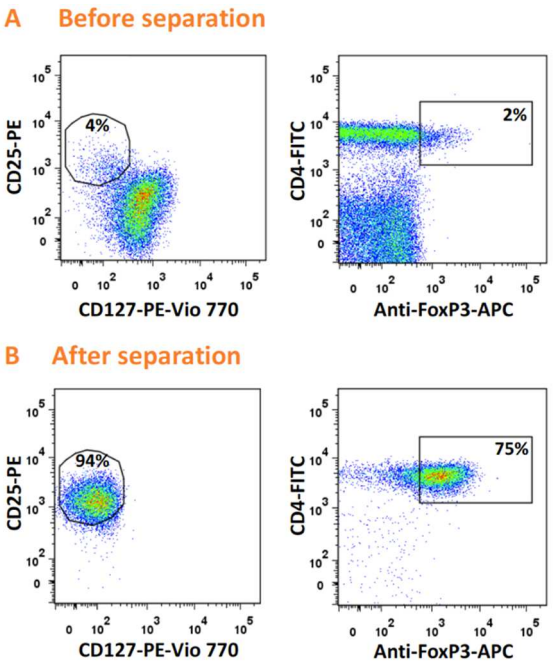

Figure 1: Flow cytometry analysis of Treg cells.
$CD4^+CD25^+CD127^{dim/-}$ regulatory T cells were isolated from human PBMCs by using the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II, human. The cells were fluorescently stained with CD4-FITC, CD25-PE and CD127-PE-Vio 770 and Anti-FoxP3-APC before (A) and after (B) separation.

4. *In vitro* expansion of regulatory T cells using the Treg Expansion Kit

Prepare the expansion medium as described in section "Material preparation". The Treg Expansion Kit is based on MACSiBead™ Particles preloaded with CD3 and CD28 antibodies. Best expansion of Treg cells is accomplished by using MACSiBead Particles and Treg cells at a bead-to-cell ratio of 4:1.

4.1. Preparation of Treg cells

1. Determine the total number of Treg cells.

2. Wash Treg cells by adding 5–10 volumes expansion medium to the cells and centrifuge at 300×g for 10 minutes. Discard supernatant.

3. Resuspend cells at a concentration of 1×10^6 cells/mL in expansion medium. Pipet 100 µL ($1x10^5$ Treg cells) in each well of a 96-well round-bottom plate.

4.2. Preparation of CD3/CD28 MACSiBead™ Particles

▲ The CD3/CD28 MACSiBead™ Particles have a concentration of 2×10^7 beads/mL and should be used at a bead-to-cell ratio of 4:1.

▲ MACSiBead Particles are bigger in size than MACS® MicroBeads and sediment rapidly. It is therefore mandatory to bring the MACSiBead Particles into suspension by vortexing prior to use.

1. Resuspend CD3/CD28 MACSiBead Particles thoroughly by vortexing and transfer 4 times the amount of Treg cells to a suitable tube. (e.g. for expansion of $1x10^6$ Treg cells, transfer $4x10^6$ (=200 µL) of MACSiBead Particles).

2. Wash MACSiBead Particles by adding 300–600 µL of culture medium and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.

3. Resuspend CD3/CD28 MACSiBead Particles in expansion medium to get a final concentration of 2×10^7 beads/mL (e.g. when $4x10^6$ of MACSiBead Particles are used, resuspend in 200 µL of expansion medium).

4.3. Co-cultivation of Treg cells and MACSiBead Particles

1. Add 20 µL of CD3/CD28 MACSiBead Particles to each well containing the Treg cells. As the MACSiBead Particles sediment rapidly, the bead suspension should be vortexed from time to time prior addition to the well.

2. The final volume of each well is now 120 µL. Let the co-culture of Treg cells and MACSiBead Particles rest for one day at 37°C and 5–7% CO_2.

4.4. Expansion process

Day 1: Add 100 µL of expansion medium to each well.

Day 3-5 (depending on medium color): Either split Treg cells (including MACSiBead Particles) at a ratio of 1:2 and resuspend cells in 200 µL of fresh expansion medium or carefully aspirate 100 µL of old medium and add 100 µL of fresh expansion medium.

Day 5-8: Either transfer Treg cells (including MACSiBead Particles) to a 24-well plate in higher volumes, e.g., 5×10^5 Treg cells in 500 µL, or split Treg cells (including MACSiBead Particles) at a ratio of 1:2 and resuspend cells in 200 µL of fresh expansion medium.

Day 14: The Treg expansion process can be stopped here or proceeded by restimulation of Treg cells. If you want to stop Treg expansion here, determine the total cell number and the frequency of $CD4^+$FoxP3-expressing cells by performing a surface and intracellular flow cytometry staining to assess the result of expansion. For details refer to section 3. If you want to proceed with restimulation the CD3/CD28 MACSiBead Particles may be removed prior restimulation. For details refer to section 5.

5. Bead removal and restimulation

Removal of MACSiBead Particles may be required before restimulation with MACSiBead Particles. In addition, bead removal is required before magnetic separation of cells with MACS MicroBeads or before stimulation with different agents or antigens.

5.1. Removal of MACSiBead Particles

1. Harvest cells, transfer to an appropriate tube and wash once with separation buffer.

2. Determine cell number and resuspend cells in separation buffer at a density of up to 2×10^7 cells/mL and vortex thoroughly.

3. Place the tube in the magnetic field of the MACSiMAG™ Separator (fig. 2). Detailed instructions on how to use the MACSiMAG Separator can be found in the MACSiMAG Separator datasheet.

4. Allow the MACSiBead Particles to adhere to the wall of the tube:
   5 mL tubes: 2 minutes
   15 mL or 50 mL tubes: 4 minutes

5. Retaining the tube in the magnet, carefully remove the supernatant containing the MACSiBead Particles-depleted cells and place in a new tube.

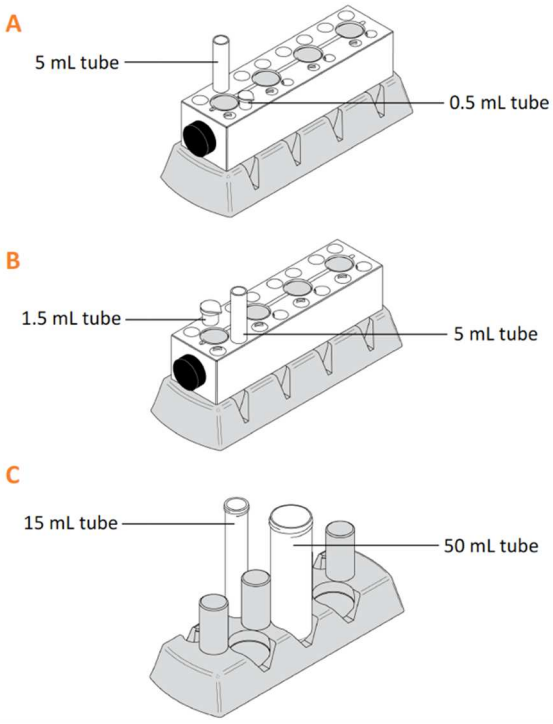

Figure 2: The MACSiMAG Separator. (A) MACSiMAG Separator with tube rack positioned for tubes of 5 mL or 0.5 mL in size. (B) MACSiMAG Separator with tube rack positioned for tubes of 1.5 mL or 5 mL in size. (C) MACSiMAG Separator with 15 mL and 50 mL tube.

6. Remove the tube from the separator and add separation buffer to reach the same volume as before.

7. Vortex sample, replace tube in the MACSiMAG Separator and repeat steps 4–5.

8. Collected cells can now be further processed as required, e.g., for restimulation with MACSibead Particles.

5.2. Restimulation of Treg cells

▲ Restimulation is performed with a bead-to-cell ratio of 4:1.

Depending on Treg number and resulting volumes, the restimulation can either be performed in a 96-well plate or in a 24-well plate. If you prefer to restimulate Treg cells in a 96-well-plate, refer to section 4 and follow the protocol. Alternatively, restimulation may also be performed in 24-well plates in higher volumes, for example, 5×10^5 Treg cells in 500 µL of expansion medium and 100 µL of CD3/CD28 MACSiBead Particles (= 2×10^6 particles).

Day 15: Add 100 µL of expansion medium per well when restimulation is performed in a 96-well plate, or 500 µL of expansion medium per well when restimulation is performed in a 24-well plate.

Day 17-19: (depending on medium color): Either split Treg cells again (including the MACSiBead Particles) at a ratio of 1:2 and fill the well to 1 mL with fresh expansion medium or carefully aspirate 500 µL old medium and add 500 µL fresh expansion medium.

Day 21: The Treg expansion process should be stopped after 21 days. To assess the result of expansion, determine the total cell number and the frequency of FoxP3-expressing cells, by performing a surface and intracellular flow cytometry staining. For details see section 3.

▲ **Note:** Bead removal is required before certain downstream applications, i.e. magnetic separation of cells with MACS MicroBeads or before stimulation with different agents or antigens. For details refer to section 5.1.

5.3. Flow cytometry analysis

To determine the frequency of FoxP3-positive $CD4^+$ cells after expansion, the cells were analyzed by flow cytometry.

1. Lymphocytes were identified according to FSC and SSC (data not shown).

2. Dead cells were excluded from the analysis by using the live/dead cell exclusion marker PI (data not shown).

3. Lymphocytes were further analyzed for their expression of CD4 (y-axis) and FoxP3 (x-axis) to assess the frequency of FoxP3-expressing $CD4^+$ cells among lymphocytes (fig. 3A). Please note that CD4 is used here to identify Treg cells as CD25 is not an appropriate Treg marker after expansion as it might also be upregulated on activated T cells.

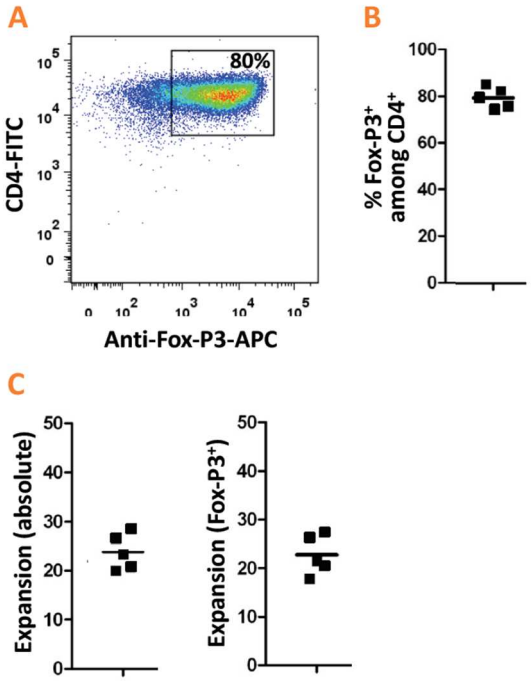

Figure 3: Analysis of Treg expansion. $CD4^+CD25^+CD127^{dim/-}$ regulatory T cells were isolated from human PBMCs by using the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II, human and expanded for 21 days with the Treg Expansion Kit. Afterwards, the cells were fluorescently stained with CD4-FITC and FoxP3-APC antibodies. (A) Representative FACS plot of expanded Treg cells and (B) frequency of $FoxP3^+$ Treg cells among $CD4^+$ cells after expansion from five experiments. (C) Fold-expansion of total cells (left) and $FoxP3^+$ cells (right) (Fold-expansion comparing the initial and the final cell number). Each dot represents one experiment.