

Chapter 2

Polyclonal Expansion of Human CD4⁺CD25⁺ Regulatory T Cells

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Abstract

Based on results from experimental animal models, the adoptive transfer of CD4⁺CD25⁺FOXP3⁺ regulatory T cells (Treg) is expected to be efficacious in treating autoimmune and inflammatory diseases, as well as in preventing alloresponses after solid organ or stem-cell transplantation. For potential clinical applications, large numbers of Treg cells in maximum purity will be required to avoid the risk of disease exacerbation by contaminating effector T cells. We have recently described methods for the efficient in vitro expansion of human Treg cells and identified CD4⁺CD25^{high}CD45RA⁺ T cells as the ideal starting population for the generation of homogeneous and stable Treg cell products. Here, we provide detailed instructions for their identification, isolation, expansion, and functional characterization.

Key words: Immunotherapy, Tolerance, Suppressor T cell, Transplantation, T-cell therapy

1. Introduction

Thymus-derived CD4⁺CD25⁺Foxp3⁺ regulatory T cells play a crucial role in the maintenance of peripheral self-tolerance, as loss-of-function mutations of the *foxp3* gene abrogate their suppressive activity and cause fatal autoimmunity in mice and humans (1). Similarly, the deletion of Foxp3⁺ Treg cells causes autoimmune syndromes in rodents but strengthens immune responses to tumors (2) or to microbial infections (3). Inversely, their adoptive transfer protects from autoimmunity and exacerbated inflammatory responses in various disease models (4). In experimental colitis, transferred Treg cells even cure ongoing disease (5). These findings highlight the relevance of Treg cells for physiological immune reactions and raised interest in their therapeutic manipulation.

Treg-based immune modulation is particularly attractive in the context of allogeneic transplantation. Several groups could show that they contribute to tolerance induction after solid organ transplantation (6) and the coinfusion of donor-derived Treg cells protects from lethal graft-versus-host disease (GVHD) after allogeneic stem-cell transplantation (SCT) (7). As allogeneic SCT is per se a cellular therapy, both in terms of replacement of the myeloid compartment and with regard to the beneficial graft-versus leukemia effect (GVL) mediated by donor T cells, this treatment modality seems ideally suited for the exploration and exploitation of cellular immune suppression. In murine SCT experiments, the coinfusion of large numbers of freshly isolated donor Treg cells [at a 1:1 ratio with conventional T cells (Tconv)] prevented GVHD without abrogating GVL effects (8, 9). Clinical trials exploring this strategy are now ongoing at our and several other institutions and good manufacturing practice (GMP)-compatible isolations strategies have been developed for that purpose (10). Yet, it has to be emphasized that such isolation strategies were specifically designed for the prevention of GVHD in SCT, where the cotransplantation of Tconv cells is intended for the prevention of opportunistic infections and the promotion of GVL effects. Thus, these technologies purposely enrich Tregs to only 50–60% purity (10) [and unpublished data] but were explicitly not developed for the treatment of GVHD or any other diseases, as a contamination of the transferred cell product with effector T cells might aggravate the respective condition and endanger patients (11). In our opinion, a therapeutic Treg cell product has to fulfil several criteria: (1) Clinically relevant cell numbers have to be achieved reliably; therefore, efficient in vitro expansion of Treg cells is required. (2) Maximum purity of Treg cells, as determined by current knowledge and technology, has to be enforced (currently evaluated by the level of FOXP3 expression). (3) No proinflammatory cytokines should be secreted, as this might contribute to disease progression. To address these issues, we previously described efficient in vitro expansion protocols that permit the 2–3 log expansion of CD4⁺CD25^{high} Tregs within 2–3 weeks. Although human and murine Treg cells were described to be anergic and therefore unsuited for large-scale expansion, we showed that the supplementation of high-dose IL-2, combined with TCR- and CD28-mediated stimulation, was sufficient to promote their in vitro proliferation (12). Cross-linking of anti-CD3 and anti-CD28 antibodies either coupled to magnetic beads or presented by Fc-receptor expressing fibroblasts turned out to be important for their expansion as was supplementation of sufficient IL-2, due to their inability to produce this vital cytokine by themselves (13). However, once FOXP3 staining reagents became available, we discovered that only a portion of CD4⁺CD25^{high}

T cells (50–70%) maintained expression of this lineage-defining transcription factor during in vitro culture (14). When we further examined this subpopulation, we found that those cells also constitutively expressed CD62L and CCR7, suggesting that they originated from either naïve or central memory-type cells (15). Using CD45RA as a discriminating marker, we were able to identify the CD45RA⁺ subpopulation of Treg cells as their precursors (14). Treg cell cultures derived from this starting population homogeneously maintained FOXP3 expression, showed maximum suppressive activity in vitro, and did not secrete any proinflammatory cytokines. In contrast, the CD45RA⁻ subpopulation of CD4⁺CD25^{high} Treg cells lost FOXP3 expression over time, started to secrete cytokines, and showed diminished in vitro suppressive activity (14). In mouse, the stability of FOXP3 expression is at least in part determined by epigenetic mechanisms (16). In particular, DNA demethylation of a conserved noncoding region with known enhancer function in the *FOXP3* locus is required for stable FOXP3 expression (termed TSDR for Treg-specific demethylated region) (17). We have recently confirmed that human Treg cells are also demethylated in this TSDR (18). However, whereas DNA methylation in this region increases in CD45RA⁻ Treg cells after repetitive stimulation, CD45RA⁺ Treg cells maintain the demethylated state of their TSDR during in vitro culture (19). Thus, based on phenotypic, functional, molecular, and epigenetic data, CD45RA⁺ Treg cells seem to be the most reliable source for the generation of a homogeneous Treg cell product described to date. Even recently published alternative isolation strategies, such as depletion of CD127⁺ cells (20, 21) or selection of CD49d⁻ cells (22) (and unpublished results), do not ensure the same quality of a Treg cell product after prolonged in vitro expansion (23). Thus, until we possess specific pharmacological agents that exclusively target Treg cells to enhance their suppressive activity in vivo, the isolation and expansion of CD45RA⁺ Treg cells seem the most promising strategy for the generation of safe and homogeneous Treg cell products for therapeutic purposes. Unfortunately, appropriate technologies for the reliable and GMP-compatible isolation of these rare Treg cell subpopulations are not yet available for clinical trials. However, several groups now collaborate with biotech and pharmaceutical companies for the implementation of such treatment strategies. Until then, the difficulties during the development of appropriate isolation and expansion techniques should not seduce us to employ insufficient technologies, since the health and safety of patients remains the prime priority of our efforts. With those principles in mind, we provide below detailed instructions for the identification, isolation, expansion, and functional characterization of Treg cell populations.

2. Materials

2.1. MACS- and FACS-Based Isolation of T Cell Subpopulations

1. Anti-PE and anti-CD4 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany).
2. MACS buffer: PBS containing 1% FCS, 2 mM EDTA.
3. MACS Midi magnets and LS columns.
4. MACS Pre-Separation Filters (Mesh size: 30 μ M; Miltenyi Biotec).
5. FACS staining buffer: PBS containing 2% FCS.
6. Propidium iodide (SIGMA-Aldrich, Steinheim, Germany; final concentration: 2.5 μ g/mL) for exclusion of dead cells in flow cytometry; kept as sterile 10 \times solution in PBS at 4°C; added immediately before sorting or analysis.
7. FACS Aria® high-speed cell sorter (BD Biosciences).
8. Antibodies used for cell isolation:

Antibody	Fluorescent dye	Clone	Source
CD 4	FITC ; PE-Cy7	SK3	BD Biosciences
CD 25	PE ; PE-Cy7; APC	2A3	BD Biosciences
CD 45 RA	APC ; Pacific Blue	MEM-56	Invitrogen
CD 45 RA	FITC	HI100	BD Pharmingen
CD 127	PE	hIL-7R-M21	BD Pharmingen

The color combination used routinely for isolation of naïve CD4⁺CD25⁺CD45RA⁺ Treg cells is indicated in bold (see also Note 1).

2.2. Cell Culture and Quality Control Assays

1. RPMI 1640 (without l-glutamine), supplemented with 2 mM L-glutamine, 10% FCS, 1% MEM vitamins, 1 mM sodium pyruvate, 1% MEM NEAA, 10 mM HEPES, 100 U/mL penicillin, 100 μ g/mL streptomycin, 50 μ M 2-mercaptoethanol. This medium is used for the maintenance of L929 feeder cells and for suppression assays. cRPMI for Treg cell expansion cultures is further supplemented with 300 U/mL human IL-2 (PROLEUKIN S, Novartis Pharma GmbH, Nürnberg, Germany).
2. 96-well and 24-well flat bottom plates (BD Falcon™, BD Biosciences, Heidelberg, Germany).
3. 96-well round bottom plates and cell culture flasks (25 and 75 cm²; Costar®, Corning, New York, NY).
4. PBS with 0.05% Trypsin, 0.02% EDTA (PAN, Aidenbach, Germany).

5. PBS (w/o Ca²⁺ and Mg²⁺; PAA, Pasching, Germany).
6. NA/LE purified antibodies for cell culture:
Anti-CD3: OKT3 (JANSSEN-CILAG GmbH, Neuss, Germany).
Anti-CD28: CD28.2 (BD Pharmingen).
7. Magnetic beads used for in vitro expansion of T-cell subpopulations:
Dynabeads[®] Human T-Activator CD3/CD28 (111.31D; Invitrogen).
8. Dynal magnets for bead removal (e.g., MPC[™]-L or MPC[™]-15).
9. Phorbol 12-myristate 13-acetate (PMA; SIGMA-Aldrich) is dissolved at 2 mg/mL in DMSO and stored in aliquots at -20°C.
10. Ionomycin (Alexis, Lausen, Switzerland) is stored as 1 mM solution in DMSO at -20°C.
11. CFSE (carboxyfluorescein succinimidyl ester; SIGMA-Aldrich Cat No.:21888) is stored in aliquots as 5 mM stock solution in DMSO at -20°C.
12. FOXP3 staining kit (includes fixation/permeabilization buffers and PE-, APC-, or Pacific Blue-labeled anti-FOXP3 antibody; clone:PCH101 [eBioscience, San Diego, CA]).

3. Methods

3.1. Isolation of Human CD4⁺CD25^{high} Treg Cells

1. PBMC are isolated from leukapheresis products of healthy volunteers (after their informed consent and in accordance with protocols approved by the local authorities) by density gradient centrifugation over Ficoll/Hypaque (Biocoll, Biochrom AG, Berlin, Germany).
2. Cells are stained with PE-anti-CD25 (10×10⁶ cells/5 µL PE-anti-CD25/100 µL FACS buffer) for 20 min at 4°C, followed by incubation with anti-PE magnetic beads (Miltenyi Biotec) in MACS buffer and positive selection of CD25⁺ cells on LS columns according to the manufacturer's instructions.
3. Isolated cells are washed once in FACS buffer and stained with FITC-anti-CD4 (4×10⁶ cells/10 µL FITC-anti-CD4/100 µL FACS buffer) for 20 min at 4°C.
4. Stained cells are washed once, filtered through a 30 µm mesh filter, and resuspended in FACS buffer at a cell density of 10–20×10⁶ cells/mL.

5. Cells are sorted on a FACS Aria® high-speed cell sorter into 5-mL- or 15-mL tubes filled with 200 μ L or 500 μ L FCS, respectively. Gating strategy see Fig. 2. Sterile PI is added immediately before sorting.
6. Sorted cells are centrifuged at $350\times g$ for 10 min, resuspended in cRPMI, counted, and can be used either directly or after short-term expansion in functional assays (see Note 2).

**3.2. Isolation
of Human
CD4⁺CD25⁺CD127⁻
Treg Cells**

1. As in Subheading 3.1.
2. Cells are incubated with anti-CD4 magnetic beads (Miltenyi Biotec) in MACS buffer and CD4⁺ cells are positively selected on LS columns according to the manufacturer's instructions.
3. Isolated cells are washed once in FACS buffer and stained with FITC-anti-CD4, PE-anti-CD127, and APC-anti-CD25 (4×10^6 cells/10 μ L FITC-anti-CD4/5 μ L PE-CD127/2 μ L APC-anti-CD25/100 μ L FACS buffer) for 20 min at 4°C.
4. Steps 4–6: As in Subheading 3.1. For Gating strategy see Fig. 1 (see Note 3).

**3.3. Isolation
of Human
CD4⁺CD25^{high}CD45RA⁺
Naïve Treg Cells**

1. As in Subheading 3.1.
2. As in Subheading 3.1.
3. Isolated cells are washed once in FACS buffer and stained with FITC-anti-CD4 and APC-anti-CD45RA (4×10^6 cells/10 μ L FITC-anti-CD4/1.5 μ L APC-anti-CD45RA/100 μ L FACS buffer) for 20 min at 4°C.
4. Steps 4–6: As in Subheading 3.1. For Gating strategy see Fig. 2 below.

**3.4. In Vitro Expansion
of Human CD4⁺CD25⁺
Treg Using Murine
L929 Fibroblasts**

**3.4.1. Maintenance
of the HuCD32⁺ Murine
Fibroblast Cell Line L929**

1. Cells of the L 929-derived murine Ltk⁻ cell line stably transfected with human Fc γ RII (CD32) (24) are grown in 25 or 75 cm² flasks in cRPMI to subconfluent monolayers.
2. To harvest the cells, medium is discarded; trypsin/EDTA is added (1–2 mL for 25 cm² flask, 3–3.5 mL to 75 cm² flask) and incubated at 37°C (incubator) for 5 min.
3. Cells are detached by thorough tapping against the side wall of the flask.
4. 10 mL of cRPMI (5 mL for 25 cm² flask) are added and cells are thoroughly mixed.
5. Cells are transferred to a 50-ml cell culture tube (BD Falcon™); the tube is filled with cRPMI and centrifuged at $300\times g$ for 10 min.
6. Pelleted cells are resuspended in cRPMI, counted, and new flasks are set up with 0.25 to 1×10^6 cells/20 mL cRPMI/75 cm² flask.

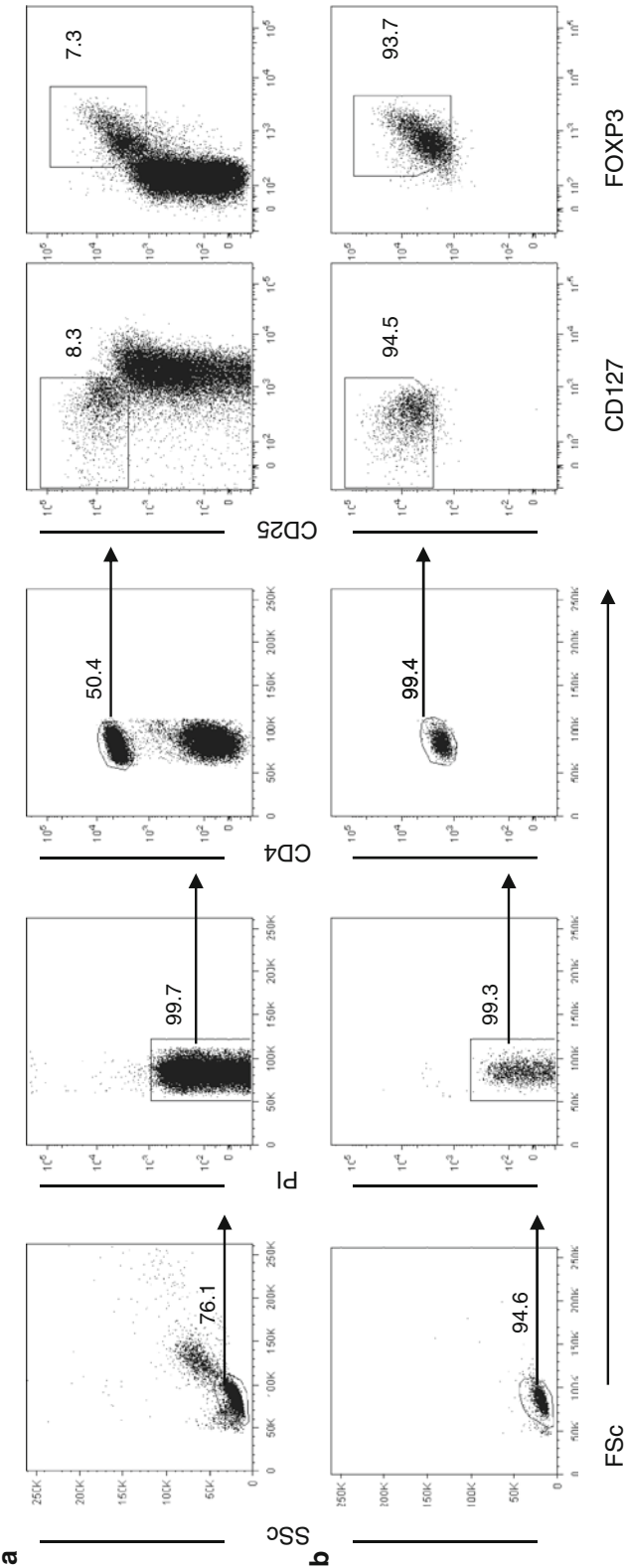


Fig. 1. Isolation of CD4⁺CD25⁺CD127⁻ Treg cells (a) Gating strategy for, and FOXP3 expression of, CD4⁺CD25⁺CD127⁻ Treg cells within human PBMC. (b) Reanalysis of CD4⁺CD25⁺CD127⁻ Treg cells, isolated according to the protocol detailed in Subheading 3.2.

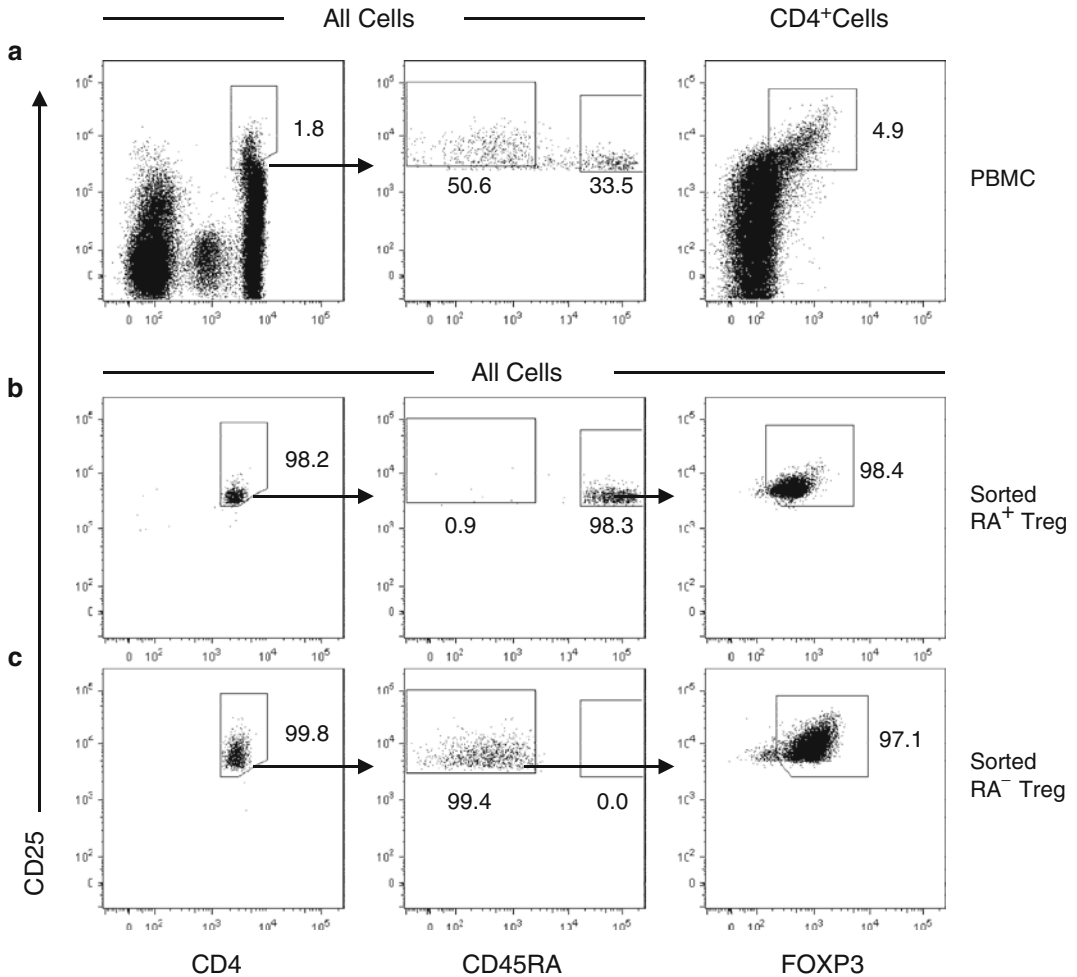


Fig. 2. Isolation of CD4⁺CD25⁺CD45RA⁺ and CD4⁺CD25⁺CD45RA⁻ Treg cells. **(a)** MNC from a leukapheresis product are stained with FITC-anti-CD4, PE-anti-CD25, and APC-anti-CD45RA. To isolate the entire CD4⁺CD25^{high} Treg population (as mentioned in Subheading 3.1), the gate is set on the population with a CD4 expression level slightly lower than that of the CD4⁺CD25^{int} T cell population (representing recently activated Tconv cells) and with a CD25 expression level above that of the main CD4⁺CD25⁺ population (representing mostly activated B cells). To further separate Treg cells into a naive (CD45RA⁺) and a memory-type (CD45RA⁻) subpopulation, gates are set as shown in the middle panel. **(b, c)** Reanalysis of sorted CD4⁺CD25⁺CD45RA⁺ **(b)** and CD4⁺CD25⁺CD45RA⁻ Treg cells **(c)**. In parallel, MNC as well as aliquots of the sorted populations are stained with FITC-anti-CD4, PE-anti-CD25, APC-anti-CD45RA, and Pacific Blue-anti-FOXP3 to identify Treg cells (and verify successful sorting) via FOXP3 expression (see Notes 4 and 5).

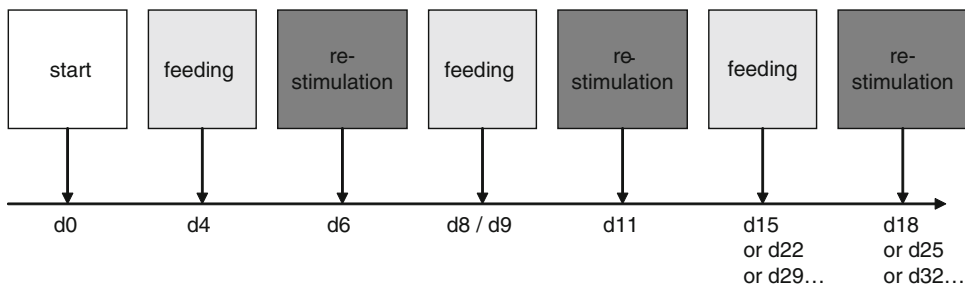
3.4.2. Preparation of HuCD32⁺ L929 Feeder Cells

1. HuCD32⁺ L929 cells are harvested, washed once, counted, and cell number is adjusted to 5×10^6 cells/mL in cRPMI
2. Cells are irradiated with 70 Gy.
3. Immediately after irradiation, cells are diluted to appropriate cell concentration in cRPMI and seeded to cell culture plates:
 - 15,000 cells/100 μ L in 96-well flat bottom plates.
 - 80,000 cells/400 μ L in 24-well plates.
 - 380,000 cells/2.5 mL in 6-well plates.

**3.4.3. Setup
and Maintenance of Treg
Cell Cultures on L929
Feeder Cells**

4. Cells are allowed to adhere (approx. 60 min at 37°C) before T cells are added. Feeder cells can be prepared 1 day prior to T-cell culture setup.

1. 96-well flat bottom plates with adherent feeder cells in cRPMI are prepared (see Subheading 3.4.2, step 2).
2. Sorted CD4⁺CD25⁺ Treg cells or subpopulations thereof are seeded at 10,000 cells/well in 100 µL cRPMI, supplemented with 600 U/mL IL-2, 200 ng/mL OKT-3, and 200 ng/mL anti-CD28 (resulting in final concentrations for IL-2, OKT-3, and anti-CD28 of 300 U/mL, 100 ng/mL, and 100 ng/mL, respectively).
3. Cells are incubated at 37°C and 5% CO₂ in humidified atmosphere.
4. On day 4, 100 µL/well is removed and replaced by fresh cRPMI with 300 U/mL IL-2.
5. On day 6, cells are harvested; cells from parallel cultures are united, washed once in cRPMI, counted, and reseeded to either 24-well or 6-well plates at 100,000 cells and 500,000 cells/well in a final volume of 500 µL and 3 mL, respectively. IL-2, OKT-3, and anti-CD28 are added to result in final concentrations as stated in step 2.
6. Timeline for the maintenance of long-term Treg/feeder cell cultures:



7. Routinely obtained expansion rates for FACS-purified human CD4⁺CD25⁺CD45RA⁺ Treg cells kept on L929 feeder cells for up to 25 days are shown in Fig. 3.

**3.5. In Vitro Expansion
of Human Treg Cells
Using Antibody-
Coated Bead
Stimulation**

1. Sorted Treg cells in cRPMI/300 U/mL IL-2 are mixed with Dynabeads® Human T-Activator CD3/CD28 to result in a bead:cell ratio of 4:1.
2. 10,000 cells/100 µL/well are seeded in 96-well round bottom plates. The plates are kept at 4°C for approx. 1 h before culture setup to minimize adherence of the seeded cells to the walls of the well and thereby facilitate cell accumulation at the bottom of the well.

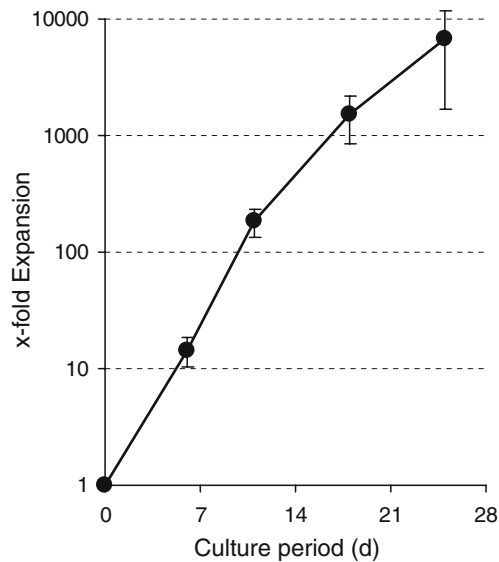


Fig. 3. Expansion of CD4⁺CD25⁺CD45RA⁺ Treg cells on huCD32⁺ L929 feeder cells. CD4⁺CD25⁺CD45RA⁺ Treg cells were sorted from 11 different leukapheresis products and cultured for up to 25 days on huCD32⁺ L929 feeder cells as detailed in Subheading 3.4. Cells were harvested and counted on indicated days using Trypan Blue for dead cell exclusion. Cumulative expansion rates were calculated. Data represent mean ± SD from 11 individual cultures.

3. Cells are incubated at 37°C and 5%CO₂ in humidified atmosphere.
4. On day 4, 100 µL cRPMI with 300 U/mL IL-2 are added to each well.
5. On day 7, cells are resuspended within the wells (cells during the first week of in vitro culture tend to stick together tightly and can only be singularized while still in the well), cells from parallel wells are united in a 5-mL or 15-mL cell culture tube, thoroughly resuspended, and placed in a magnet holder for 2–3 min.
6. Cells are removed from the tubes, transferred to a new tube, counted, washed once with cRPMI, and resuspended at 5 × 10⁵/mL in cRPMI/300 U/mL IL-2 with stimulatory beads added to result in a bead:cell ratio of 1:1.
7. 1 mL/well of the cell and bead mixture is added to the wells of a 24-well plate.
8. On day 11, 1 mL cRPMI/300 U/mL IL-2 is added to each well. If necessary, wells can be split into two and refilled to 1 mL final volume with cRPMI/300 U/mL IL-2.
9. On day 14, cells are harvested and restimulated as detailed in step 6 (see Note 6).
10. Timeline for the maintenance of long-term Treg/Bead cultures

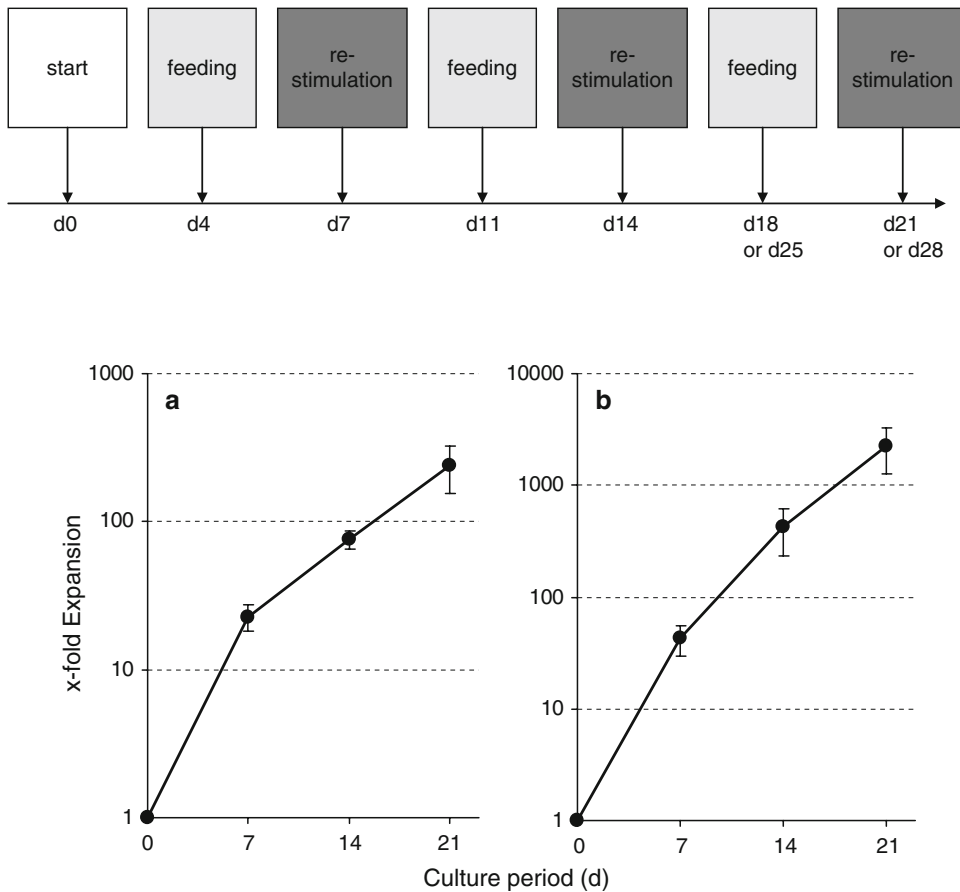


Fig. 4. Expansion of CD4⁺CD25⁺CD45RA⁺ Treg using antibody-coated magnetic beads. CD4⁺CD25⁺CD45RA⁺ Treg cells were sorted from four different leukapheresis products and cultured for up to 21 with anti-CD3/anti-CD28-coated paramagnetic beads as detailed in Subheading 3.5. Cells were harvested and counted on indicated days with Trypan Blue for exclusion of dead cells. Cumulative expansion rates were calculated. Data represent mean \pm SD from four individual cultures.

11. Routinely achieved expansion rates for FACS-purified CD4⁺CD25⁺CD45RA⁺ Treg cells cultured with anti-CD3/anti-CD28 coated stimulatory beads are shown in Fig. 4.

3.6. Quality Control During and at End of Culture

3.6.1. Staining of In Vitro Expanded Treg Cells for FOXP3 with or without Simultaneous Staining for Proinflammatory Cytokines

FOXP3 expression is still the most widely used and generally accepted quality control criterion for in vitro-expanded Treg cells. However, since FOXP3 can also be transiently expressed by activated Tconv cells, a short resting period before staining for FOXP3 is recommended. Alternatively, expanded cells can be simultaneously stained for FOXP3 and proinflammatory cytokines to reveal any cells converting from a Treg to a Tconv phenotype during in vitro culture.

1. Expanded cells are harvested (in case expansion was done with stimulatory beads, the beads are removed), washed once in cRPMI, and reseeded at 1×10^6 cells/mL in cRPMI/300 U/mL IL-2 in 6-well plates (5 mL/well) or 24-well plates (1 mL/well) for 2–4 days (see Note 7).
2. *Variation 1.* Cells are stained directly for FOXP3 (can be combined with surface staining for CD4 and/or CD25) following the manufacturer's instructions.

Variation 2. Cells are washed once with cRPMI, reseeded at 1×10^6 cells/mL in cRPMI in 24-well plates (1 mL/well), and stimulated with PMA (20 ng/mL)/ionomycin (1 μ M) in the presence of monensin (GolgiStop; BD Biosciences; 0.67 μ L/mL) for 5 h. Cells are washed once and are now ready for combined FOXP3/intracellular cytokine staining. A common combination would be CD4 (surface staining performed before fixation/permeabilization), FOXP3, IL-2 and/or IFN- γ (anti-cytokine and anti-FOXP3 antibodies are added simultaneously; the protocol for FOXP3 staining should be strictly applied). Typical results with both quality control staining protocols are shown in Fig. 5.

*3.6.2. Functional Analysis
of In Vitro Expanded Treg
Cells: Suppression
of Autologous Tconv Cells
After Polyclonal Stimulation*

Quality control of in vitro expanded Treg cells can also be carried out by determination of their suppressive activity after polyclonal activation. Commonly, proliferation of freshly isolated or cryo-preserved autologous Tconv cells is used as readout system.

Since in vitro expanded Treg cells show a higher tendency than freshly isolated Treg cells to proliferate under normal assay conditions, a CFSE dilution assay is preferable to detection of ³H-thymidine incorporation for quantification of the proliferative capacity of cocultured Tconv cells.

1. Cryopreserved PBMC are thawed and CD4⁺CD25⁻ Tconv cells are isolated by either MACS- or FACS-based techniques as detailed above.
2. All CFSE labeling steps should be done with the lights in the laminar flow turned off.
3. 10^7 Tconv cells in a 15-mL conical tube are washed twice with PBS (w/o FCS!) and resuspended in 500 μ L PBS (see Note 8).
4. 500 μ L of a 2 \times CFSE solution (4 μ M in PBS) is added and cells are incubated for 4 min. at room temperature in the dark.
5. Cells are washed twice with PBS/10% FCS or cRPMI, counted, and adjusted to 1×10^6 /mL in cRPMI.
6. In vitro expanded and 2–4 days-rested Treg cells are washed once and adjusted to 1×10^6 cells/mL in cRPMI. If Treg cells are to be titrated, 1:2 serial dilutions are prepared.

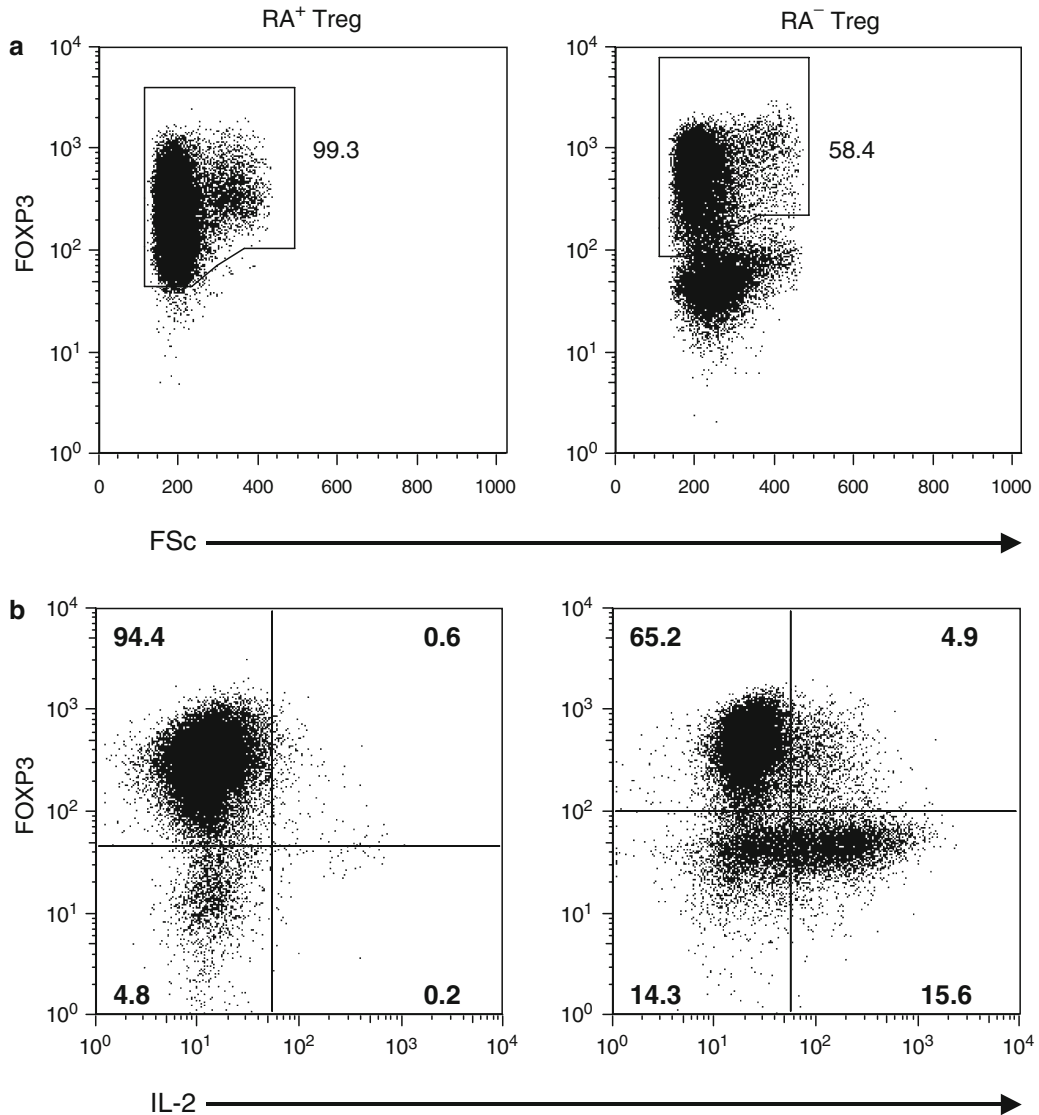


Fig. 5. FOXP3 and intracellular cytokine expression in in vitro expanded CD45RA⁺ and CD45RA⁻ Treg cells. **(a)** CD4⁺CD25⁺CD45RA⁺ and CD4⁺CD25⁺CD45RA⁻ Treg cells were isolated by MACS/FACS, cultured in vitro on L929 feeder cells for 2 weeks and rested for 4 days in cRPMI/300 U/mL before staining for FOXP3. **(b)** Cells prepared and treated as in **(a)** were stained for FOXP3 and intracellular IL-2 after an additional 5 h-stimulation period with PMA/ionomycin in the presence of monensin.

7. Antigen-presenting cells (APC) are prepared from CD4-depleted PBMC (step 1) by incubation with anti-CD2 beads (e.g., Dynabeads[®] CD2 Pan T; Invitrogen) following the manufacturer's instructions. CD2-depleted cells are washed once in cRPMI, irradiated with 30 Gy, and adjusted to 2×10^6 cells/mL in cRPMI.

8. Cultures are set up in 96-well round bottom plates in the following order (see also Notes 9–11):
 - 50 μ L APC (\rightarrow 100,000 cells/well).
 - 50 μ L OKT3 (400 μ g/mL \rightarrow 100 μ g/mL final conc.).
 - 50 μ L Tconv cells (\rightarrow 50,000 cells/well).
 - 50 μ L Treg cells (\rightarrow a maximum of 50,000 cells/well).
9. Cultures are kept at 37°C, 5% CO₂ in a humidified atmosphere for 3 days.
10. Cells are harvested, those from parallel wells are united, washed once in FACS staining buffer, stained, for example, with CD4 and CD25, and analyzed by FACS.

4. Notes

1. Staining for CD25 should not be performed with FITC-labeled antibodies, as discrimination of CD25^{high} and CD25^{int} CD4 T cell populations is difficult due to low resolution. Also, we obtained better results with clone 2A3 as compared to clone M-A251 (both BD Biosciences).
2. As detailed in the introduction, bulk CD4⁺CD25^{high} Treg cells comprise CD45RA⁺ (naïve) as well as CD45RA[−] (memory-type) cells. Both represent Treg cell populations, as demonstrated by phenotypic and epigenetic analysis. However, since they differ considerably in Treg phenotype stability during in vitro culture, Treg cells isolated solely on the basis of CD4 and CD25 expression should only be used directly or after short-term in vitro activation/expansion.
3. Since the CD4⁺CD25⁺CD127[−] Treg cell population also comprises naïve as well as memory-type cells, the same caution should be exercised in applying these cells as detailed above for bulk CD4⁺CD25⁺ Treg cells.
4. Another characteristic of Treg cells that can be used for their identification and flow-cytometric isolation in combination with staining for surface markers is their smaller size (hence the lower CD4 expression mentioned in Fig. 2), resulting in a lower forward scatter signal.
5. CD45RA and CD45RO expression levels are inversely correlated, with most CD4 T cells expressing both isoforms of the common leukocyte antigen (CD45) to a certain degree. However, only CD4⁺ Treg cells with a high CD45RA expression level are also homogeneously positive for the two additional developmental markers CCR7 and CD62L, which are usually associated with a naïve phenotype and have been

- shown by us before to identify those Treg cells with a stable suppressor/regulatory phenotype during in vitro cultivation.
6. The bead:cell ratio of 4:1 in the first week and then 1:1 for all subsequent restimulation rounds is optimized for the needs of Treg cells, but are too strong for the expansion of conventional (CD4⁺CD25⁻) T cells (Tconv). For those cells, a 1:1 ratio for the first week and a 1:10 ratio thereafter turned out to be more successful.
 7. In vitro expanded T cells with a stable Treg phenotype (e.g., CD4⁺CD25⁺CD45RA⁺ Treg cells) do not form clusters when kept under these conditions and show only very little proliferation. In contrast, in vitro-expanded Tconv cells tend to aggregate in small clusters and retain considerable proliferative capacity even after removal of the TCR stimulus (e.g., antibody-coated beads).
 8. Volume can be adjusted to actual cell number but should be no less than 100 μ L.
 9. Several negative and positive controls should be included in CFSE assays: (a) cultures with APC and CFSE-labeled Tconv w/o OKT3 (\rightarrow useful for exact identification of undiluted CFSE label intensity as most of the Tconv cells will survive but not proliferate under these conditions), (b) in vitro-expanded CD4⁺CD25⁻ T cells should be added instead of Treg cells to discriminate Treg-cell-specific suppression from unspecific effects of in vitro culture-adapted cells.
 10. CD8⁺ instead of CD4⁺ T cells can also be used as responders in Treg suppression assays. This facilitates discrimination of strongly proliferating (and thus CFSE-losing) responder cells from unlabeled Treg cells.
 11. A minimum of five parallel wells/intended FACS staining combination is recommended.

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