Video Article

In Vitro Differentiation of Human CD4$^+$FOXP3$^+$ Induced Regulatory T Cells (iTregs) from Naïve CD4$^+$ T Cells Using a TGF-β-containing Protocol

Angelika Schmidt[1], Szabolcs Éliás*[1], Rubin N. Joshi*[1], Jesper Tegnér[1]

[1]Unit of Computational Medicine, Center for Molecular Medicine, Department of Medicine Solna, Karolinska Institutet, Karolinska University Hospital, & Science for Life Laboratory

*These authors contributed equally

Correspondence to: Angelika Schmidt at schmidt_angelika@outlook.com

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Abstract

Regulatory T cells (Tregs) are an integral part of peripheral tolerance, suppressing immune reactions against self-structures and thus preventing autoimmune diseases. Clinical approaches to adoptively transfer Tregs, or to deplete Tregs in cancer, are underway with promising first outcomes.

Because the number of naturally occurring Tregs (nTregs) is very limited, studying certain Treg features using *in vitro* induced Tregs (iTregs) can be advantageous. To date, the best although not absolutely specific protein marker to delineate Tregs is the transcription factor FOXP3. Despite the importance of Tregs including non-redundant roles of peripherally induced Tregs, the protocols to generate iTregs are currently controversial, particularly for human cells. This protocol therefore describes the *in vitro* differentiation of human CD4+FOXP3+ iTregs from human naïve T cells using a range of Treg-inducing factors (TGF-β plus IL-2 only, or their combination with retinoic acid, rapamycin or butyrate) in parallel. It also describes the phenotyping of these cells by flow cytometry and qRT-PCR.

These protocols result in reproducible expression of FOXP3 and other Treg signature genes and enable the study of general FOXP3-regulatory mechanisms as well as protocol-specific effects to delineate the impact of certain factors. iTregs can be utilized to study various phenotypic aspects as well as molecular mechanisms of Treg induction. Detailed molecular studies are facilitated by relatively large cell numbers that can be obtained.

A limitation for the application of iTregs is the relative instability of FOXP3 expression in these cells compared to nTregs. iTregs generated by these protocols can also be used for functional assays such as studying their suppressive function, in which iTregs induced by TGF-β plus retinoic acid and rapamycin display superior suppressive activity. However, the suppressive capacity of iTregs can differ from nTregs and the use of appropriate controls is crucial.

Video Link

The video component of this article can be found at https://www.jove.com/video/55015/

Introduction

CD4+CD25+FOXP3+ regulatory T cells (Tregs) suppress other immune cells and are critical mediators of peripheral tolerance, preventing autoimmunity and excessive inflammation[1]. The importance of Tregs is exemplified by the human disease immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), in which loss of Tregs due to mutations in the `master´ Treg transcription factor forkhead box P3 (FOXP3) leads to severe systemic autoimmune disease, lethal at an early age. However, Tregs act as a double-edged sword in the immune system as they can also hamper anti-tumor immunity in certain settings[2]. Therapeutic manipulation of Treg number and function is therefore subject to numerous clinical investigations. In cancer, depletion of Tregs can be desirable and some success of clinical approaches encourages further research[3]. In autoimmune and inflammatory diseases, in addition to therapeutic effects of Tregs in several mouse disease models, recent first in-man trials of adoptive Treg transfer to prevent graft-*versus*-host disease (GvHD)[4-7] and to assess safety in treating type 1 diabetes[8] showed very promising outcomes.

Naturally occurring Tregs (nTregs) comprise thymic-derived tTregs and peripherally induced pTregs, with non-redundant essential functions in maintaining health[9-11]. However, nTreg numbers are limited, encouraging the complementary approach of inducing Tregs (iTregs) *in vitro* from naïve T cell precursors[12]. Still stability of iTregs, presumably due to lack of demethylation in the so-called Treg-specific demethylated region (TSDR) in the FOXP3 gene locus[13], remains a concern and several studies indicate that *in vivo* induced Tregs are more stable[14].

To date, FOXP3 remains the best protein marker for Tregs but it is not absolutely specific because human conventional CD4+CD25- T cells transiently express intermediate levels of FOXP3 upon activation[15,16]. Although significant progress has been made in elucidating the regulation of FOXP3 expression, much remains to be discovered regarding the induction, stability and function of FOXP3 particularly in human cells. Despite differences to nTregs, *in vitro* induced FOXP3+ $CD4^+$ T cells can be used as a model system to study molecular mechanisms of FOXP3 induction and as a starting point to develop protocols in the future that allow for generation of iTregs that are more similar to *in vivo* generated Tregs, which could be applicable for adoptive transfer strategies in the future.

There is no `gold standard´ protocol to induce human iTregs, and current protocols have been developed based on mimicking Treg-inducing conditions *in vivo*: interleukin 2 (IL-2) and transforming growth factor β (TGF-β) signaling are crucial for FOXP3 induction *in vivo*[17], and all-trans retinoic acid (ATRA) — which is produced *in vivo* by gut-associated dendritic cells — is frequently used to enhance FOXP3 induction *in vitro*[18-21]. We have developed additional human Treg-inducing protocols using butyrate[22], a gut microbiota-derived short-chain fatty acid that was recently shown to augment murine Treg induction[23,24]. We also recently established a new protocol for generation of iTregs with superior suppressive function *in vitro* by using a combination of TGF-β, ATRA and rapamycin[22], the latter being a clinically approved mammalian target of rapamycin (mTOR) inhibitor that is known to promote FOXP3 maintenance during human Treg expansion[25,26].

This method describes the reproducible *in vitro* generation of human CD4+FOXP3+ iTregs using a set of different conditions, and their subsequent phenotyping by flow cytometry and quantitative reverse transcription polymerase chain reaction (qRT-PCR) to reveal protocol-specific patterns of expression of FOXP3 and other Treg signature molecules such as CD25, CTLA-4, EOS, as well as repression of IFN-γ and SATB1 *expression*[22]. The generated cell populations can be used for functional assays regarding suppressive activity or for molecular studies, either concerning general FOXP3 regulators or to study effects specific to certain compounds such as butyrate or rapamycin. Further understanding of molecular mechanisms driving Treg differentiation is highly relevant for future therapeutic approaches in autoimmunity or cancer to specifically target molecules involved in Treg generation and function.

Protocol

Human peripheral blood mononuclear cells (PBMCs) were freshly isolated from anonymized healthy donor buffy coats purchased from the Karolinska University Hospital, Sweden. Ethical permit for the experiments was obtained from the Regional Ethical Review Board in Stockholm (Regionala etikprövningsnämnden i Stockholm), Sweden (approval number: 2013/1458-31/1).

1. T Cell Isolation from Peripheral Blood

1. **PBMC Isolation**
    1. Pre-lay 15 ml density centrifugation medium (such as Ficoll) solution in 50 ml tubes (5 tubes per buffy coat). Pre-warm to room temperature.
    2. Fill up buffy coat with phosphate buffered saline (PBS) (room temperature) to 180 ml. If fresh blood is used, dilute blood with equal volume of PBS.
    3. Tilt tube to the side and slowly overlay density centrifugation medium with ~35 ml diluted blood per tube. Add the blood very carefully, without any mixing of the density centrifugation medium phase and blood layer.
    4. Centrifuge 20 min at 1,150 x g at room temperature without brake. Operate the centrifuge without brake and if possible with low to medium acceleration to prevent mixing of the layers.
    5. Take out the white ring containing PBMCs between the density centrifugation medium and the plasma phase. Transfer to a new 50 ml tube (1 time with 5 ml pipette and 2 times with 2 ml plastic-Pasteur pipette). Take as little as possible plasma and density centrifugation medium. Do not touch the red erythrocyte pellet.
    6. Wash PBMCs. Split the PBMCs into 50 ml tubes (4 tubes per buffy coat). Fill each to 50 ml with PBS.
    7. Centrifuge 450 x g for 10 min at room temperature.
    8. Remove and discard supernatant, pool the cells into 1 tube with 10 ml RPMI/10% fetal calf serum (FCS) medium and resuspend well. If cell clumps are present, strain cells through a 40 µm cell strainer.
    9. Transfer cells in T175 cell culture flask for adherent cells. Rinse tubes with 40 ml medium and combine with cells (50 ml total). If needed, remove an aliquot for flow cytometry analysis (see step 1.4). Usually, $CD4^+$ T cells comprise about 30-40% of cells in the lymphocyte gate, and naïve T cells comprise about 30 to 60% of $CD4^+$ T cells depending on the donor.
    10. Incubate cells in laying flask for 45 to 90 min at 37 °C/5% CO_2. This step will deplete monocytes by plastic adherence. It is not mandatory, but will increase the percentage T cell yield per amount of PBMCs in the following steps.
    11. Resuspend cells (in the 50 ml medium contained in the flask) and rinse cell layer on the bottom of the flask well. Distribute cells equally into two 50 ml tubes (the previous tubes may be re-used for the same donor). Rinse flask with 50 ml fresh RPMI/10% FCS medium, and transfer equally into the same 2 tubes.
        NOTE: PBMCs may be stored overnight at 4 °C with tubes laying on the side, or ideally, directly used for T cell isolation.

2. **nTreg Isolation by Magnetic-activated Cell Sorting**
    1. Prepare isolation buffer: 0.5% (w/v) human serum albumin and 2 mM EDTA in PBS. Always keep buffer at 4 °C. Alternatively to human albumin, use bovine albumin.
    2. Resuspend and count PBMCs with an automatic or manual cell counter in the presence of trypan blue (do not count small platelets). If cell clumps are observed, strain cells through a 40 µm cell strainer.
    3. Centrifuge PBMCs at 450 x g for 10 min at room temperature.
    4. Resuspend cells (with 1 ml pipet) in 90 µl isolation buffer per 10^7 PBMCs to obtain a single cell suspension. Keep cells in the original 50 ml tubes (2 tubes per buffy coat).
    5. Add 2 µl CD25 beads per 10^7 cells, mix, and incubate for 15 min at 4 °C.
    6. Fill with PBS to 30 ml. Centrifuge at 450 x g for 10 min at 4 °C.
    7. Prepare LS columns (2 per buffy coat): equilibrate with 3 ml isolation buffer, and discard flow through. Tubes can be re-used to rinse further columns.

8. Resuspend cells in 3 ml isolation buffer per tube (with 1 ml pipet). Transfer cells to LS column, and collect flow through in fresh 15 ml tubes.
9. Rinse the 50 ml tubes with 2 ml Isolation buffer each. Transfer rinsing buffer to the columns.
10. Wash column 2x with 3 ml Isolation buffer each. Always wait until the column stopped dripping, before adding any new liquid. Store flow through at 4 °C for later steps.
11. Remove column from magnet. Elute 2x with 3 ml isolation buffer per column into 15 ml tube.
    NOTE: The eluate from 2 columns per donor can be combined. Do not dip the column into the liquid.
12. Prepare and equilibrate new LS columns (1 column per buffy coat). Transfer the eluate from 1.2.11 to the column. Flow through can be discarded. After eluate has passed through the column, rinse tube and wash column 3x with 3 ml isolation buffer.
    NOTE: The second column is crucially needed to increase purity.
13. Remove column from magnet. Elute $CD25^{++}$ "nTregs" 2x with 3 ml Isolation buffer.
14. Centrifuge at 450 x g for 10 min at 4 °C. Remove supernatant completely.
15. Wash cells with 15 ml T cell culture medium, centrifuge at 450 x g for 10 min at 4 °C. Remove supernatant completely.
16. Resuspend cells in 0.5 ml T cell culture medium, supplemented with 100 IU/ml IL-2, each. Transfer cells to 24-well plate. Rinse tube with 0.5 ml medium (+ 100 IU/ml IL-2) and combine into 24-well plate.
17. Count nTregs (as in step 1.2.2). The yield is usually about 1-4 x 10^6 Tregs per buffy coat. Adjust density of Tregs to 1-2 x 10^6 cells/ml with T cell culture medium + 100 IU/ml IL-2.
18. Incubate Tregs at 37 °C/5% CO_2 until use.

3. **Naïve $CD4^+$ T Cell Isolation by Magnetic-activated Cell Sorting**
   NOTE: Take flow through from step 1.2.8-1.2.10 to proceed with cell isolation. Alternatively, if no nTregs were isolated, proceed directly from PBMCs.
   1. Combine 2 tubes per donor of Treg-depleted PBMCs in 50 ml tube. Fill with PBS to 50 ml at room temperature.
   2. Centrifuge PBMCs at 200 x g for 5-10 min at room temperature. Remove and discard supernatant. Resuspend cells in 50 ml PBS.
   3. Repeat step 1.3.2 twice.
      NOTE: The PBS washes are crucial for removal of platelets, which will not be removed with the following negative isolation kit. Platelets remain in the supernatant at this low-speed centrifugation. Platelet depletion can be monitored in the individual steps on a microscopic slide. The steps have to be performed with PBS and centrifugations at room temperature.
   4. Resuspend PBMCs in 50 ml PBS. Count cells as in step 1.2.2.
   5. Centrifuge PBMCs at 450 x g for 10 min at 4 °C. Discard supernatant and resuspend cells in 40 µl Isolation buffer (4 °C) per 10^7 cells.
   6. Add 10 µl of Naïve $CD4^+$ T Cell Biotin-Antibody Cocktail II per 10^7 cells.
   7. Mix, and incubate for 10 min at 4 °C.
   8. Wash cells by adding 40 ml PBS (4 °C), and centrifuge at 450 x g for 10 min at 4°C. Remove supernatant and resuspend cells in 80 µl isolation buffer per 10^7 cells.
   9. Add 20 µl of anti-biotin microbeads per 10^7 cells. Mix well, and incubate for 15 min at 4 °C.
   10. Fill to 50 ml with cold PBS, and centrifuge 450 x g for 10 min at 4°C.
   11. Prepare LS columns (equilibrate with 3 ml isolation buffer). To avoid column overload, use one column for maximally 250 x 10^6 PBMCs, or less if PBMCs contain many red blood cells.
   12. Remove supernatant and resuspend cells in 3 ml isolation buffer. Transfer cells to LS column. Collect flow through, which contains the naïve $CD4^+$ T cells, in 15 ml tubes.
   13. Rinse tube with 2 ml isolation buffer. Transfer to the column.
   14. Wash column 3x with 3 ml isolation buffer. Always wait until the column stops dripping, before adding any new liquid.
   15. Take the flow through (naïve $CD4^+$ T cells) and centrifuge 450 x g for 10 min at 4 °C. Columns can be discarded.
   16. Remove supernatant completely. Wash cells with 15 ml medium, centrifuge 450 x g for 10 min at room temperature and remove supernatant completely.
       NOTE: Isolation buffer contains EDTA, which chelates calcium ions and thus impairs T cell activation. Before any stimulation assays, remove the isolation buffer completely and wash cells twice with 15 ml medium.
   17. Resuspend cells in T cell culture medium to 2-3 x 10^6 cells per ml. Rest cells in appropriate flask or well over night in the incubator. If cells are used directly for stimulation assays, wash them one more time with medium.

4. **Purity Control Staining**
   1. Take ~50,000 cells (Tnaïve; nTreg; PBMCs) each, in 20 µl.
      NOTE: If both Tnaïve and nTreg panel are to be stained, take 2 samples each.
   2. Prepare 2x concentrated antibody premix (in PBS) as appropriate for the instrument, for example: Tnaïve panel: CD4-PerCP 1:5; CD45RA-FITC 1:10, CD45RO-PE 1:5, CD8-eFlour450 1:8. For Treg panel, replace CD45RO-PE with CD25-PE, 1:10.
      NOTE: Only certain CD25 antibodies, that recognize different epitopes than the CD25-microbeads, can be used to stain nTregs isolated with CD25 microbeads.
   3. Add 20 µl antibody premix to 20 µl cells. Also prepare single stainings for each fluorochrome (using a sample containing positive cells, *e.g.*, PBMCs) and an unstained sample, for flow cytometry settings and compensation.
   4. Incubate for 15 min at room temperature in the dark.
   5. Fill each sample with 200 µl PBS, and centrifuge 450 x g for 5-10 min.
   6. Take up cells in fluorescence activated cell sorting (FACS) buffer (= isolation buffer without EDTA) and analyze by flow cytometry. The purity of naïve $CD4^+$ T cells is usually >95% (see **Figure 2**).

2. Treg Induction Culture

1. **Preparation of Stimulation Media and Plates**
   1. Prepare and pre-warm T cell culture medium: serum-free hematopoietic medium supplemented with 2 mM L-alanyl-L-glutamine.

2. Prepare cytokine, stimulation antibody and compound stock concentrations.
    1. Prepare IL-2 stock solution: 400,000 IU/ml in sterile-filtered (with acid-resistant filter) 100 mM acetic acid (167 µg/ml if activity of the used lot is 2,400 IU per 1 µg; confirm with provider). Aliquot to sterilized low-protein binding 0.5 ml tubes, seal with Parafilm, freeze on dry ice and store at -80 °C for long-term storage or -20 °C for up to 3 months.
    2. Prepare TGF-β1 stock solution (for 10 µg vial, carrier-free): Centrifuge TGF-β1 powder (1,000 x g 3 min), add 100 µl of 4 mM HCl (sterile-filtered with acid-resistant filter) to prepare stock solution with 100 µg/ml, allow to dissolve completely; vortex. Aliquot 5 µl each to sterilized low-protein binding 0.5 ml tubes, seal with Parafilm, freeze on dry ice and store at -80 °C for up to 6 months.
    3. Prepare ATRA stock solution: Dissolve powder at 20 mg/ml (66.6 mM) in DMSO. Prepare stock solution of 10 mM by further dilution in DMSO and store aliquots, protected from light, at -80 °C for up to 1 year. ATRA is sensitive to light, heat and air.
    4. Prepare rapamycin stock solution: 1 mg/ml (1.11 mM) in DMSO, store in aliquots at -80 °C for up to 1 year.
    5. Prepare sodium butyrate stock solution: 0.908 M (0.1 mg/ml) stock in sterile ultra-pure H_2O and sterile filter. Aliquot and store at -20 °C.

3. Prepare 4x concentrated premixes (50 µl per well) in T cell culture medium as in **Table 1**.
4. On the previous day, coat plates with anti-CD3 antibody. Coat desired number of wells from 96 U-well plate with 5 µg/ml anti-CD3 antibody in PBS, 65 µl/well. Wrap plate with foil and incubate overnight at 4 °C.
   NOTE: Do not use the margin wells of 96 well plates. Bigger wells may be used, but U-shaped wells facilitate use of the same cell numbers per area across experiments. If more cells are needed, pool the required amount of wells after culture. Usually, after 4-6 days of expansion, 2 wells each sample are sufficient for flow cytometry analysis and RNA analysis.
    1. On the day of plating, shortly before plating, remove anti-CD3 solution from wells. Fill wells with 200 µl/well PBS. Remove PBS. Repeat washing with 200 µl/well PBS. Remove PBS completely and use plates immediately.

5. Prepare plates (do not use margin wells):
    1. For each well, add 50 µl of 4x anti-CD28/IL-2 premix (except for "unstimulated" cells, add T cell culture medium), 50 µl 4x TGF-β1 premix (for all iTregs) or 50 µl T cell culture medium for "stimulation only" mock control, 50 µl 4x ATRA, ATRA/rapamycin or butyrate premix (where applicable) or medium
    2. Fill all empty wells, including the margin wells, with 200 µl PBS. Pre-warm plates at 37 °C/5% CO_2.

2. **Prepare Naïve T Cells for Plating**
    1. After resting cells, transfer to 15 ml tube, rinse flask/wells with pre-warmed T cell culture medium and pool to tube. Fill tube to 15 ml with T cell culture medium.
    2. Centrifuge cells at 450 x g for 10 min at room temperature.
    3. Remove medium and take up cells in fresh, pre-warmed T cell culture medium to a density of 2.2-2.6 x 10^6/ml. Count cells as in step 1.2.2 and adjust density if needed.
    4. Add cells to prepared stimulation plates (see 2.1), 50 µl cells/well (110,000-130,000 cells/well). Each well should now contain a total volume of 200 µl.
    5. Wrap plates in aluminum foil to protect ATRA from light; incubate at 37 °C/5%CO_2 for desired time periods.

3. **Monitor Treg Induction Culture**
    1. Monitor Treg cultures by light microscopy (40X magnification). From about day 2 to 3, small clusters of proliferating cells should become visible, which merge into bigger clusters at later time points. Cultures grown with rapamycin generally grow less. See also **Figure 3**.
    2. At desired time points, take samples for RNA extraction or flow cytometry phenotyping (see below). For later time points with cell proliferation, 1 well each is sufficient, otherwise take 1 x 10^5-1 x 10^6 cells each for RNA and 3 x 10^5-1 x 10^6 cells for flow cytometry.

4. **Assessing FOXP3 Stability**
    1. For assessing stability of the iTreg phenotype as described previously[22,27], wash iTregs twice with T cell culture medium, and culture iTregs further in T cell culture medium either without or with anti-CD3 and anti-CD28 restimulation as described in 2.1 and 2.2. Add cytokines, such as 50 IU/ml IL-2, to study their influence on FOXP3 stability.
    2. At desired time points, take samples for RNA extraction, flow cytometry phenotyping (see below), and TSDR methylation analysis as described[22,28].

3. Phenotypic Analysis of iTregs by qRT-PCR

1. **Preparation of Samples**
    1. At desired time points, take 1 x 10^5 to 1 x 10^6 cells per sample for RNA extraction. Transfer cells to an RNase-free 1.5 ml tube, and centrifuge at 500 x g for 5 min.
    2. If needed, remove part of supernatant and freeze for enzyme-linked immunosorbent assay (ELISA) or similar analysis. Remove remaining supernatant completely from cells. Proceed directly to RNA extraction or freeze cell pellet on dry ice and store at -20 °C to -80 °C for up to 2 weeks.
    3. Extract RNA and perform qRT-PCR for *FOXP3* and a housekeeping gene (such as *RPL13A*) according to standard protocols. In addition, measure expression of other Treg signature genes (for example `Treg up´ genes *IL2RA*, *CTLA4*, *IKZF4*, and `Treg down´ genes *IFNG, SATB1*).

4. Phenotypic Analysis by Flow Cytometry

NOTE: This protocol is optimized for staining in 96U well plate format with 3 x 10^5-1 x 10^6 cells. If there are less cells per well, start with several wells and pool as described below.

1. **Cell Restimulation (Optional)**
    1. If staining for intracellular cytokines is desired, pulse cells with Phorbol 12-myristate 13-acetate (PMA)/Ionomycin in the presence of a protein transport inhibitor.
    NOTE: This procedure does not influence FOXP3 expression in above-described iTreg cultures, but other markers may change — for example, CD4 is downregulated.
    2. Prepare 10x Brefeldin A/PMA/Ionomycin premix (20 µl per well) in T cell culture medium: Brefeldin A stock solution 1:100, Ionomycin (stock 5 µg/µl) 1:1,300, PMA (stock 12.35 µg/µl, pre-dilute 1:1235) 1:100 of pre-diluted stock.
    3. 4 hours before staining, add 20 µl Brefeldin A/PMA/Ionomycin 10x mix per well to obtain end concentrations of 1:1,000 Brefeldin A, 0.5 µM (375 ng/ml) Ionomycin, 10 ng/ml PMA.
    4. Incubate for 4 hours at 37 °C/5%CO_2

2. **Surface Staining**
    NOTE: The panel suggested here is a suggestion; other panels may be used depending on the antigens of interest and instrument setup.
    1. Cool PBS on ice. Prepare FACS buffer (PBS with 0.5% human serum albumin, HSA) and cool on ice. BSA or FBS may be used as an alternative to HSA.
    2. Re-plate cells in staining plate: Resuspend cells with a pipette and transfer the cells into a new 96U well plate, leaving one well free around each well (to prevent spillover in later staining procedure). If less than desired cell numbers are present in the original well, remove part of the supernatant before resuspension, and pool several wells into a staining well.
    3. Centrifuge plate 400 x g for 10 min at room temperature.
    4. Remove the supernatant. Pour out supernatant into waste box, leave the plate upside down and immediately (without turning back the plate) tap the plate once on absorbent paper. Then immediately turn back the plate. Vortex plate to resuspend cells in remaining liquid.
    5. Add 25 µl surface staining premix (CD25-PE 1:20, CD4-PerCP 1:5 in FACS buffer) and resuspend.
    NOTE: Also include single stainings for each of the used fluorochromes with samples that contain positive cells for the respective antigen; and include an unstained sample. These will be needed for instrument compensation setup. Alternatively, use compensation beads.
    6. Incubate 30 min at 4 °C in the dark.
    7. Add 200 µl PBS, centrifuge plate 400 x g for 10 min at 4 °C and remove supernatant as described in step 4.2.4. Vortex plate.
    8. Repeat step 4.2.7 twice.

3. **Viability Staining**
    NOTE: Viability staining is indispensable, since after fixation/permeabilization dead cells cannot sufficiently be excluded by fsc/ssc and may give rise to unspecific signals. A fixable viability dye has to be used.
    1. Resuspend cells in 130 µl viability stain premix (fixable viability dye-eFlour780 1:1,400 in PBS) and immediately resuspend cells with the multichannel pipet.
    NOTE: Also include a single staining for the viability dye containing dead cells, *e.g.,* unstimulated T cells cultured for several days or kill cells by applying heat as by the manufacturers' instructions. These will be needed for compensation.
    2. Incubate 30 min at 4 °C in the dark.
    3. Centrifuge plate at 400 x g for 10 min at 4 °C. Remove supernatant as in step 4.2.4. Vortex plate.
    4. Fill with 200 µl FACS buffer. Repeat step 4.3.3.
    5. Fill with 200 µl PBS. Repeat step 4.3.3.

4. **Intracellular Staining with FOXP3 Staining Buffer Set**
    1. For each well, prepare: 150 µl Fix/Perm buffer (dilute Fix/Perm concentrate 1:4 with diluent); 850 µl 1x permeabilization buffer (dilute 10x permeabilization buffer 1:10 with ultra-pure H_2O).
    2. After vortexing, resuspend cells in 150 µl Fix/Perm buffer and immediately resuspend with pipette. It is important to immediately resuspend to avoid fixation of cell clumps.
    Caution: Fixation buffer contains paraformaldehyde and should be handled and discarded appropriately.
    3. Incubate 30 min at 4 °C in the dark.
    4. Add 100 µl cold PBS. Centrifuge at 850 x g for 10 min at 4 °C.
    NOTE: From this step onwards the centrifugation speed is increased to avoid loss of cells. After fixation, cells become invisible and care should be taken to remove supernatants as described in step 4.2.4 and immediately after the centrifugation is finished to minimize loss of cells.
    5. Remove supernatant as described in step 4.2.4. Vortex plate.
    NOTE: The staining may be paused at this step; in this case, wash the cells twice with 200 µl PBS; then take up in 250 µl PBS and store at 4 °C, protected from light. The cells can be stored for few days, then proceed with permeabilization. However, optimal results are achieved when directly continued to permeabilization.
    6. After vortexing, add 200 µl Permeabilization buffer. Centrifuge at 850 x g for 10 min at 4°C. Remove supernatant as described in step 4.2.4. Vortex plate.
    7. Add 200 µl Permeabilization buffer to resuspend.
    NOTE: Here, samples can be split into intracellular staining and isotype control sample (take away half of each sample). If cell numbers are limiting, a pooled isotype sample can be prepared (take away 10-20 µl of each sample and pool). Also, samples can be taken for Fluorescence-Minus-One (FMO) controls.
    8. Centrifuge at 850 x g for 10 min at 4 °C. Remove supernatant as described in step 4.2.4. Vortex plate.
    9. Resuspend pellet in 44 µl Permeabilization buffer plus 1 µl normal mouse serum (premixed) to block unspecific binding.

NOTE: This applies if antibodies used in the following step are of murine isotype. If other antibodies, such as derived from rat, are used, include the appropriate serum for blocking (*e.g.,* rat serum).

10. Incubate at 4 °C for 15 min in the dark.
11. Add antibodies:
    NOTE: Inquire the antibody concentrations for the specific Lots. If not done in step 4.2 with antibodies against surface antigens (*e.g.*, CD4) with the respective fluorochromes, also include single stainings for each of the used fluorochromes with samples that contain positive cells for compensation.
    1. Add 15 µl antibody premix to samples (except to single stainings, unstained samples, isotype control samples and FMO controls): Premix per sample: 0.7 µl (0.35 µg) anti-Interferon gamma (IFN-γ)-FITC (if applicable after restimulation), 2.3 µl (0.115 µg) CTLA-4 Brilliant Violet 421, 2 µl (0.05 µg) Anti-FOXP3-APC, 10 µl PBS.
    2. To isotype control samples, add 15 µl isotype antibody premix, using the same amounts of antibody as for the antibodies used in 4.4.11.1: Premix per sample: 0.7 µl (0.35 µg) mouse IgG1 K isotype control FITC (if applicable), 0.58 µl (0.115 µg) mouse IgG2a-BV421 isotype, 0.5 µl (0.05 µg) mouse IgG1 K isotype control APC, 13.2 µl PBS.
    3. For FMO controls, add antibodies as in 4.4.11.1, replacing one antibody each with PBS.
12. Incubate at 4 °C for 30 min in the dark.
13. Add 200 µl Permeabilization buffer. Centrifuge, remove supernatant and vortex as in step 4.4.8. Repeat once.
14. Resuspend cells in cold FACS buffer (volume according to the instrument used) and acquire, ideally immediately, on the flow cytometer.

Representative Results

Figure 1 shows a scheme of the experimental setup. **Figure 2** shows a representative purity control staining for magnetically isolated naïve $CD4^+$ T cells and nTregs.

F**igure 3A** shows the flow cytometry gating strategy and **Figure 3B** shows representative FOXP3 and CD25 flow cytometry stainings on day 6 of culture under the indicated iTreg or control conditions. Upon *in vitro* stimulation, most cells upregulate CD25, which is reduced in the presence of rapamycin. Only under addition of iTreg-inducing factors, a clear population of FOXP3+ cells becomes apparent, which is also enriched within $CD25^+$ cells under iTreg conditions. **Figure 3C** depicts the phenotypic appearance of iTreg cultures in the microscope with proliferating cells seen as dark clusters. Each iTreg condition shows a specific and reproducible pattern of cell proliferation: Under these culture conditions, TGF-β increases proliferation slightly, and ATRA further increases proliferation. At the same time, the inhibition of proliferation by rapamycin is apparent by the strongly reduced cluster size. The microscopic appearance of proliferation strength also corresponds to total cell counts (data not included).

Figure 4 displays representative results of qRT-PCR analyses of *FOXP3* mRNA induction in iTreg (and control) cultures at different time points. As for FOXP3 protein, *FOXP3* mRNA expression is higher in all iTregs compared to control stimulated cells, with rapamycin-treated iTregs having relatively low levels compared to other iTregs. *FOXP3* mRNA in nTregs is higher than in iTregs.

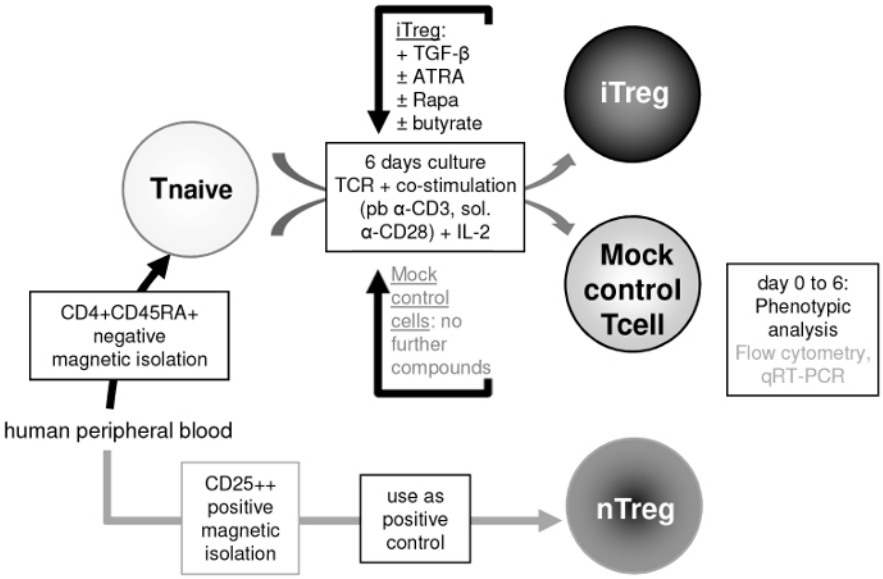

Figure 1: Experimental scheme for iTreg induction, nTreg isolation, and Treg analysis. Human naïve $CD4^+$ T cells were isolated from buffy coats and stimulated in serum-free medium with anti-CD3 and anti-CD28 antibodies plus 100 U/ml IL-2 for up to 6 days, either in the absence (`mock control cells´) or presence of different Treg-inducing factors (`iTreg´) as indicated. nTregs are isolated and assayed in parallel. Phenotypic analysis can be done by flow cytometry and qRT-PCR. Modified from Schmidt, A. *et al.* 2016[22]. Please click here to view a larger version of this figure.

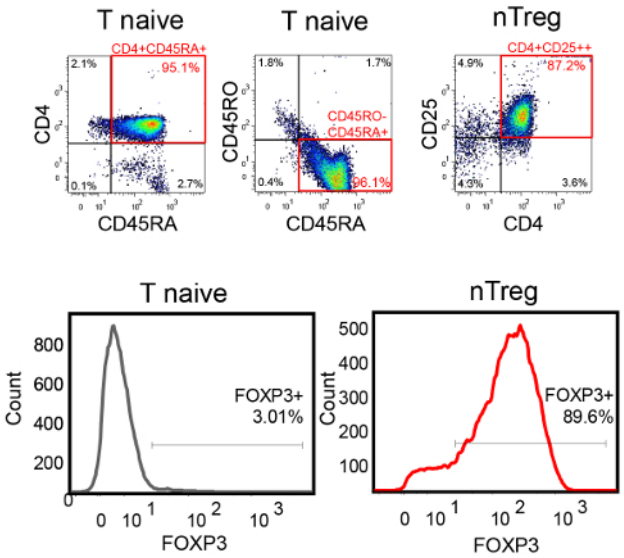

Figure 2: Cell purity of starting populations. Naïve human CD4$^+$ T cells were isolated by the Naïve CD4 T Cell Isolation Kit II, human. Upper panel: Naïve CD4$^+$ T cell purity, based on CD4, CD45RA and CD45RO, was 94 to 98% and the purity for a representative donor of more than 30 is shown (`Tnaïve´). *Ex vivo* Tregs (`nTreg´) were isolated by using limited amounts of CD25 microbeads and used as a positive control for iTreg experiments. nTreg purity of a representative donor, based on CD25 and CD4, is shown. Lower panel: Intracellular FOXP3 staining (performed as in **Figure 3** and pre-gated on live CD4$^+$ cells) is shown for naïve CD4$^+$ T cells and nTregs respectively, using cells from the same donor as in the upper panel. Modified from Schmidt, A. *et al.* 2016[22]. Please click here to view a larger version of this figure.

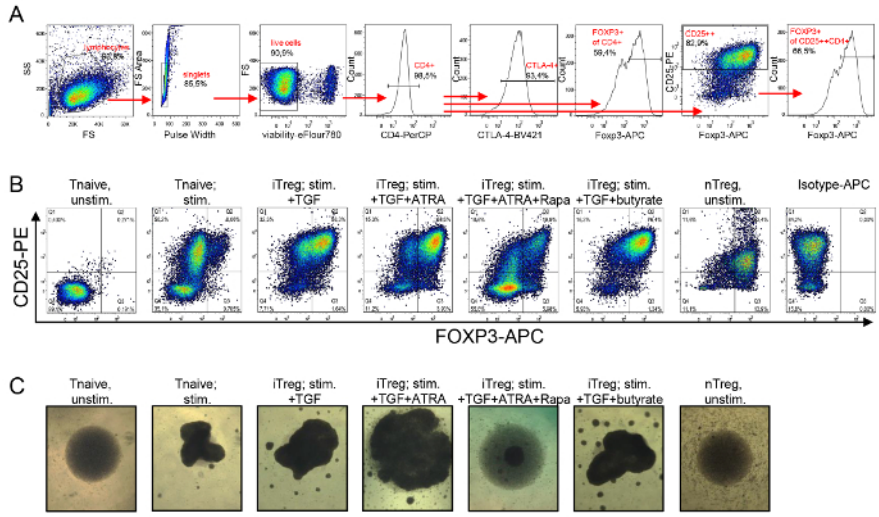

Figure 3: Phenotypic appearance of iTregs and nTregs. Human naïve CD4$^+$ T cells were cultured in serum-free medium under the indicated conditions. T cells were stimulated with anti-CD3 and anti-CD28 antibodies plus 100 U/ml IL-2 (`Stim.´). Where indicated, TGF-β1 (`TGF´), rapamycin (`Rapa´), all-trans retinoic acid (`ATRA´) or butyrate were added. nTregs (*ex vivo* isolated peripheral blood CD25^{++} cells) were left unstimulated (`unstim.´) and used as positive control. Unstimulated naïve T cells were used as negative control. (**A**) On day 6 of culture, cells were stained with surface antibodies including anti-CD25 and anti-CD4, then stained with fixable viability dye and subsequently fixed/ permeabilized and stained intracellularly with anti-FOXP3 and anti-CTLA-4 or isotype control antibodies. Acquisition and compensation was performed on a Cyan ADP flow cytometer and the data were analyzed with the FlowJo software. The gating strategy is indicated by the red arrows, and the shown example is an iTreg sample induced with TGF+ATRA. (**B**) Cells were stained as in (**A**), and FOXP3 and CD25 expression in control T cells and iTregs induced by different protocols is shown. The pseudocolor plots show representative FOXP3 and CD25 stainings for one donor, pre-gated on singlet, live CD4$^+$ cells as in (A). The isotype example is shown for an iTreg (stim.+TGF+ATRA) sample. (**C**) Representative microscopy images (40X magnification) of individual 96U-plate wells of cells cultured for 4 days. Dark clusters of cells represent proliferating cells. Please click here to view a larger version of this figure.

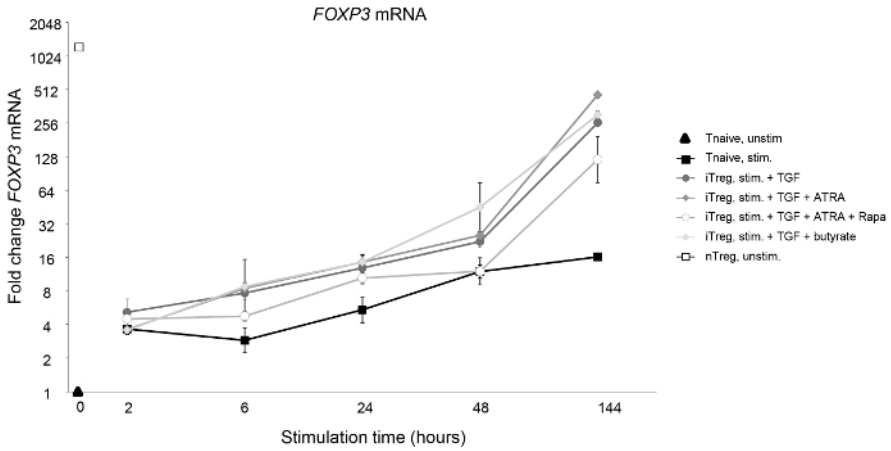

Figure 4: *FOXP3* mRNA expression upon use of different protocols for iTreg differentiation. iTregs were generated as in **Figure 3** under the indicated conditions, and at the given time points, cell samples were taken and RNA was extracted. Unstimulated naïve T cells, and unstimulated nTregs, were sampled on day 0. Samples were analyzed by qRT-PCR, and *FOXP3* mRNA expression was normalized to *RPL13A* expression for each sample. *FOXP3* mRNA expression in unstimulated naïve T cells was set to 1, and fold change of *FOXP3* mRNA was calculated. Shown are mean values and range of PCR replicate wells for a representative donor. Please click here to view a larger version of this figure.

Discussion

The described protocol enables the robust induction of human CD4+FOXP3+ iTregs from human naïve $CD4^+$ T cells. It includes a new protocol that we described recently, using a combination of TGF-β, ATRA and rapamycin, for induction of iTregs with superior *in vitro* suppressive function[22]. Compared to other published protocols, another advantage is the induction of different iTreg populations in parallel by different protocols, which enables the direct comparison of effects of certain iTreg-inducing factors, along with control cells that are activated in the presence of IL-2 alone. The described protocols enable reproducible induction of FOXP3 with low donor variation. Naïve $CD4^+$ T cells in this protocol are isolated by magnetic-activated cell sorting, but fluorescence-activated cell sorting is also possible. The expected yield of naïve $CD4^+$ T cells with this protocol is typically between 5-10%, but strongly depends on the donor (age) and appears also lower when high fractions of erythrocytes are present. If an estimate is needed, PBMCs can be stained (see step 1.4) during the monocyte depletion step. Typically, the yield of naïve $CD4^+$ T cells is about half of the "percentage naïve $CD4^+$ T cells of the lymphocyte gate" in the PBMC stain. This protocol uses limited amounts of CD25 beads to obtain CD25-high (nTreg) cells[29]. However, these are not pure Tregs, but these are just enriched in Tregs to be used as positive control. If pure Tregs are needed, other kits should be used (such as combined with CD4 enrichment and CD127-depletion) or alternatively, $CD25^+$ cells pre-enriched with 8 µl CD25 beads per 10^7 cells and stained and sorted by fluorescence-activated cell sorting with stringent CD4+CD25++ gating. Also inclusion of other markers, such as CD127 exclusion, should be considered.

It is important to consider that FOXP3 is necessary, but not sufficient to confer Treg identity[30]. While iTregs can be used to study certain aspects of, for example, FOXP3 regulation, it is important however to note that iTregs differ from nTregs in several aspects. It is therefore crucial to culture nTregs (ideally derived from the same donor) in parallel to iTregs in all assays for comparison. The difference between nTregs and iTregs is exemplified by only partial overlap of the nTreg and iTreg transcriptome as measured in murine iTregs[31]. Other Treg signature genes in addition to FOXP3 should be measured for this reason, and to ensure discrimination from activation-induced FOXP3 expression in human cells. For example, iTregs should display higher expression of CD25, CTLA-4 and EOS compared to activated T cells while expression of IFN-γ and SATB1 should be low in nTregs and iTregs induced by the protocols described here, as published previously[22]. Another important major difference between nTregs and iTregs is the lack of stable FOXP3 expression in iTregs, which most likely corresponds to methylation of the TSDR region in the FOXP3 locus in iTregs[13]. Also on a genome-wide scale, it was described that epigenetic patterns of DNA methylation and histone modifications in murine iTregs do not reflect the patterns found in nTregs[30]. We previously described that iTregs induced by the here described protocols, in contrast to nTregs, did not exhibit TSDR demethylation. Accordingly, iTregs lost FOXP3 when restimulated, but maintained FOXP3 expression when further cultured in the presence of IL-2 and without restimulation[22]. Interestingly, iTregs induced by an alternative protocol using M2 macrophage supernatants displayed enhanced FOXP3 stability, despite lack of TSDR demethylation and TGF-β being causative for FOXP3 induction[27].

Modification of iTreg-inducing protocols by addition of compounds (such as Vitamin C or hydrogen sulfide) that influence Ten-eleven Translocation (TET) methylcytosine dioxygenase enzymes, as described very recently[32-34], may add in stabilizing FOXP3 by affecting DNA methylation. Also, the stimulation strength and timing has an influence on FOXP3 expression and stability[35]. Along these lines, the use of bead-coupled CD3/CD28 antibodies instead of plate-bound antibodies was shown to increase murine iTreg *in vivo* suppressive function and stability albeit independent of TSDR demethylation[36]. Another factor that needs to be considered as a potential source of variation is the use of serum, which contains undefined factors including TGF-β which even from bovine source is 100% cross-reactive with human cells. Also, the source and activity of IL-2 can drastically influence the results of FOXP3 induction.

An important feature of Tregs is their suppressive ability, which needs to be tested subsequently with iTregs generated in this protocol. It should be noted that suppression assays with iTregs are not trivial and the literature about suppressive abilities of iTregs is controversial. Several methods and protocols to assess suppressive function of human Tregs have been published elsewhere[22,37-41], with *in vitro* proliferation assays based on flow cytometry readouts such as dilution of Carboxyfluorescein succinimidyl ester (CFSE) being the most commonly used. It is important to wash and rest iTregs before use in suppression assays, and it needs to be considered that iTregs, in contrast to nTregs, may not

be anergic but proliferate themselves during suppression assays. We consider it extremely important to use `mock´ stimulated T cells as control suppressor cells to identify the degree of unspecific suppression (such as through CTLA-4 expression, IL-2 consumption and culture overgrowth by activated T cells) that is unrelated to FOXP3+ Treg-specific effects, and the frequent lack of this control may contribute to some controversies in the literature. Using this control, we defined that only TGF-β/ATRA/Rapa-induced iTregs displayed suppressive activity *in vitro*[22]. Thus, we conclude that regarding suppressive activity (albeit not highest fraction of FOXP3+ cells), TGF-β/ATRA/Rapa is the best combination of factors of the described protocols to induce iTregs. Nevertheless, suppressive activity *in vitro* does not necessarily reflect suppressive activity *in vivo*, and indeed we determined that human iTregs generated by these protocols did not suppress in a xenogeneic graft-*versus*-host disease model at least under the conditions tested[22]. This may be related to instable FOXP3 expression which was lost in iTregs upon restimulation, in line with a lack of TSDR demethylation[22].

Depending on which aspect of iTreg features (high fraction of FOXP3+ cells, superior suppressive activity, FOXP3 stability) is most important for a particular research question, different protocols may be most suitable to study these questions, rendering it difficult to define the generally 'best' protocol for Treg induction. Furthermore, several above-described subtle experimental differences can influence results and may contribute to controversies with respect to phenotype and suppressive function of TGF-β-induced iTregs that appear between reports even with apparently similar protocols for iTreg generation[42]. For example, even within one laboratory, we observed that iTregs induced with TGF-β and IL-2 in serum-containing RPMI medium displayed some suppressive activity compared to control cells[27], while TGF-β/IL-2-induced iTregs generated in defined, serum-free T cell culture medium did not[22], despite similar levels of FOXP3.

Future applications based on these protocols should strive to further optimize Treg induction conditions to achieve a phenotype with stable FOXP3 expression, TSDR demethylation, stable phenotype without conversion to cytokine-producing effector T cells and optimal suppressive activity. Further development of iTreg induction protocols may be useful for adoptive transfer approaches in the future, in which therapy by Treg transfer is highly promising for the potential treatment of autoimmune and inflammatory diseases.

Disclosures

The authors have nothing to disclose.

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