Journal of Visualized Experiments

Generation of Human Regulatory T Cell Clones

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61075R2
Full Title:	Generation of Human Regulatory T Cell Clones
Section/Category:	JoVE Immunology and Infection
Keywords:	T cell cloning, regulatory T cells, monoclonal Treg, Treg purity, cell-based immunotherapy, Treg stability
Corresponding Author:	Johannes Nowatzky, MD New York University New York, NY UNITED STATES
Corresponding Author's Institution:	New York University
Corresponding Author E-Mail:	Johannes.Nowatzky@nyumc.org
Order of Authors:	Johannes Nowatzky, MD
	Olivier Manches
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	New York City

NYU School of Medicine
NYU LANGONE MEDICAL CENTER

Division of Rheumatology
Department of Medicine

February 19, 2020

Johannes Nowatzky, MD
Assistant Professor of Medicine
Department of Medicine, Division of Rheumatology
NYU Hospital for Joint Diseases
301 East 17th Street, Room 1611
New York, NY 10003

Phone: 212-598-6734
Fax: 212-598-6168
Email: Johannes.Nowatzky@nyumc.org

Dear Dr. Cao,

Thank you very much for your efforts and your request for another revision of our manuscript *Generation of human regulatory T cell clones*.

We have tried our best to respond to the requests put forward by you. Changes in the manuscript have been marked and a response letter is attached.

Given an upcoming NIH grant submission deadline, we would kindly like to ask you to let us know your final decision regarding acceptance or rejection of this manuscript before March 2, 2020 if this is possible. If accepted, it would be helpful for us to be able to claim in-press status by that date.

Again, it would be an honor to have the opportunity to contribute to your journal and to serve its broad readership.

Sincerely,

TITLE:

Generation of Human Regulatory T Cell Clones

AUTHORS AND AFFILIATIONS:

Johannes Nowatzky[1], Olivier Manches[2,3]

[1]Department of Medicine, Division of Rheumatology, New York University School of Medicine, New York, NY, USA

[2]Immunobiology and Immunotherapy in Chronic Diseases, Institute for Advanced Biosciences, Université Grenoble Alpes, Inserm U1209/CNRS UMR 5309, Grenoble, France

[3]Etablissement Français du Sang Auvergne-Rhône-Alpes, Grenoble, France

Corresponding Author:

Johannes Nowatzky (Johannes.Nowatzky@nyumc.org)

Email Address of Co-Author:

Olivier Manches (Olivier.Manches@efs.sante.fr)

KEYWORDS:

T cell cloning, regulatory T cells, monoclonal Treg, Treg purity, cell-based immunotherapy, Treg stability

SUMMARY:

This protocol describes the cloning and expansion of human regulatory T cells for the generation of ultra-high purity viable human Treg with stable demethylation at the Treg-specific demethylated region (TSDR) and Treg-specific phenotypic features.

ABSTRACT:

Human regulatory T cells (Treg) are notoriously difficult to isolate in high purity given the current methods of Treg enrichment. These methods are based on the identification of Treg through several activation-dependent cellular surface markers with varying expression levels in different physiologic and pathologic conditions. Populations isolated as “Treg” therefore often contain considerable numbers of non-Treg effector cells (i.e., Teff) which hamper the precise phenotypic and functional characterization of these cells, their genomic and proteomic characterization, their reliable enumeration in different states of health and disease, as well as their isolation and expansion for therapeutic purposes. The latter, in particular, remains a major hurdle, as the inadvertent expansion of effector cells homing in Treg-relevant cellular compartments (e.g., $CD4^+CD25^+$ T cells) may render Treg-based immunotherapy ineffective, or even harmful. This work presents a method that circumvents the problems associated with population-based isolation and expansion of Treg and shows that the generation of Treg candidate clones with the subsequent selection, culture, and expansion of only carefully vetted, monoclonal cells, enables the generation of an ultrapure Treg cell product that can be kept in culture for many months, enabling downstream investigation of these cells, including for possible therapeutic applications.

INTRODUCTION:

The purpose of this protocol is to enable the in vitro propagation of ultra-high purity, clonal human Treg. Isolation of Treg-enriched populations and subsequent cloning allows for the selection of desired Treg phenotypes and their expansion for further study of the biology of these cells, exploration of their potential therapeutic usefulness, and other experimental downstream applications.

Cloning Treg will yield significantly better Treg purity than polyclonal isolation and expansion approaches. This is due to the reliable, controlled exclusion of T effector cells with similar or different phenotypes from the purified population, including FOXP3-expressing ($FOXP3^{int}CD45RA^{neg}CD25^{int}$) non-Treg and $CD4^+$ $CD25^+$ $FOXP3^-$ Teff[1], among others. Clonal cell lines obtained through this approach do not face the problem of overgrowth with rapidly-expanding non-Treg clones that render very long-term expansion (i.e., several months) and in vitro culture of the cells practically impossible or at least extremely challenging. Clonal Treg also allow extensive vetting of their phenotypic features postexpansion, including through methods recognized as standard for the assessment of epigenetic features indicative of human *bona fide* natural Treg[1-4] (e.g., stable demethylation at the TSDR).

Treg expansion has mainly been performed in the form of polyclonal cell expansion for both investigative and therapeutic purposes[5-7]. The problems with Teff contamination are a major obstacle to the successful implementation of Treg cell-based immunotherapy approaches. Previous attempts to expand/generate monoclonal Treg in the literature are scarce and have failed to show maintenance of Treg features in the long term[8].

This method will be of interest to anyone studying cellular, molecular, and metabolic properties of *bona fide* human Treg. The ultrapure Treg product generated through the use of this protocol in particular lends itself to analyses using genomic approaches. Given the relatively low expansion rates that characterize human Treg in general, this method may be of limited use to those who seek the rapid expansion of massive numbers of cells. However, given the extremely high purity of the Treg generated with this protocol, smaller numbers of Treg may have similar or even better efficacy than larger expansions of polyclonal cell lines that contain effector cells that limit the overall suppressive potential of the generated product.

PROTOCOL:

This protocol follows all institutional guidelines pertaining to the ethical conduct of research involving the use of human samples. Work with human cells and other human blood products must take place at least in a BSL-2 certified environment following BLS-2 safety guidelines at a minimum.

1. Preenrichment of human peripheral blood mononuclear cells for $CD4^+CD127^{lo}CD25^{hi}$ cells

CAUTION: Use sterile technique throughout. Discard sharps immediately in an appropriate sharps container. Bleach anything that has come into contact with blood and/or blood products prior to disposal. Work in a biosafety cabinet.

1.1. Obtain human peripheral blood or blood products preenriched for human leukocytes (i.e., "leukopacs") from peripheral blood draws or leukapheresis. Process cells immediately.

NOTE: If overnight storage cannot be avoided, store and transport cells at room temperature (RT). Avoid exposure to cold.

1.2. Isolate peripheral blood mononuclear cells (PBMCs) by gradient centrifugation over density gradient medium as previously described[9].

1.3. Carefully count PBMCs using a hemocytometer or cell counter. If possible, use at least 300 x 10^6 PBMC to proceed with Treg isolation.

1.4. Resuspend PBMCs in isolation buffer (2% pooled human AB serum [PHS-AB] with 1.5 mM EDTA in phosphate buffered saline [PBS]) at a concentration of 50 x 10^6 cells/mL and proceed with magnetic sorting according to the manufacturer's instructions of the sorting kit used. For example, use magnetic cell sorting (**Table of Materials**) for negative isolation of a $CD4^+CD127^{lo}$ T cell population, followed by positive selection sorting for $CD25^+$ cells.

NOTE: A variety of products can be used for the magnetic purification of Treg, including column-based and column-free approaches. Alternatively, fluorescence-activated cell sorting (FACS) with gating on $CD127^{lo}CD25^{high}$ $CD4^+$ T cells may be performed. Complete sterility is not possible with standard FACS equipment, however, posing a significant risk of contamination. In addition, cellular stress and damage conferred by the fluidics system cannot be excluded, and the instrument lasers may impact results.

1.5. Check the resulting Treg-enriched population for purity through staining for CD4, CD3, CD127, and CD25 (**Figure 1**). Choose an anti-human CD25 antibody that recognizes a CD25 binding domain different from the one used in the sorting kit, such as the one specified in **Table of Materials**, to obtain an accurate result. Stain and fix cells using a standard surface staining protocol such as that described in Nowatzky et al.[10].

2. Cloning of Treg from a $CD127^{lo}CD25^{hi}$ preenriched human $CD4^+$ T cell suspension

2.1. Resuspend the Treg-enriched ($CD4^+CD127^{lo}CD25^{hi}$) cells obtained in step 1.4 in T cell media ([TCM]; RPMI 1640, 5% PHS-AB, 1% streptomycin/penicillin, 1% HEPES, 1% nonessential amino acids, and 1% glutamine) with 300 IU/mL of human recombinant interleukin-2 (IL-2) (**Table of Materials**) aiming for a concentration of ~1–3 x 10^6 cells/mL and count. Obtain at least three separate cell counts and calculate the average cell numbers before proceeding because accurate counts are absolutely crucial.

2.2. Prepare a single cell suspension of cells at two concentrations: (1) 3 cells/mL, and (2) 6 cells/mL. Load five round-bottom 96 well plates with 100 µL/well of suspension 1, and five plates with suspension 2. Take great care to keep cells in suspension when loading the plates to ensure a distribution of 0.3 and 0.6 cells/well, respectively.

NOTE: Do not increase the cell concentration to 1 cell/well, because this increases the risk of obtaining oligoclonal cell lines and not true clones.

2.3. Prepare feeder cells from freshly isolated human allogeneic PBMCs obtained through density gradient centrifugation as in step 1.2.

2.4. Prepare at least 10 x 10^6 feeder cells per cloning plate for irradiation by resuspending human allogeneic PBMCs in TCM without IL-2 in a 50 mL polypropylene tube at a concentration of ~10 x 10^6 PBMC/mL. Tightly close the tube and irradiate with 35 Gy in either a gamma irradiator or an X-ray based irradiation device.

NOTE: Irradiation triggers the secretion of large amounts of cytokines from the feeders that facilitate the proliferation of Teff, but may adversely affect Treg expansion. These will next be removed by washing.

2.5. Centrifuge cells at 450 x *g* and RT for 5 min after irradiation, and aspirate the supernatant. Wash cells by resuspending them in TCM without IL-2 using at least 10x the pelleted feeder cell volume of media/PBS.

NOTE: It is recommended to determine a human leukocyte antigen (HLA) expression profile of the donor cells to be cloned. This can be done through simple staining for one or several HLA types that are common in the population a respective donor is derived from (i.e., HLA-A2 or HLA-A24). PBMC used as feeder cells should not express this HLA to enable reidentification/isolation of target cells from expansion cultures prior to irradiation-induced apoptosis of the feeders.

2.6. Resuspend the irradiated and washed feeder cells in TCM with 300 IU/mL IL-2 at a concentration of 1 x 10^6 cells/mL.

2.7. Add phytohemagglutinin-L (PHA-L) (**Table of Materials**) at a concentration of 4 µg/mL (2x the required concentration in culture) and quickly proceed to step 2.8.

2.8. Add 100,000 irradiated feeder cells in 100 µL of TCM with 4 µg/mL PHA-L and 300 IU/mL IL-2 to the plated Treg-enriched cells, resulting in a total volume of 200 µL and a PHA-L concentration of 2 µg/mL in each well. Mix well by pipetting up and down 5x. Limit exposure time of the feeder cells to PHA-L before their addition to the Treg to the absolute minimum necessary, and keep in suspension until plated.

NOTE: Ensure a PHA-L concentration of 2 µg/mL in the culture at the initial seeding step, but use a lower, 1 µg/mL concentration upon subsequent expansion of Treg clones. Use 300 IU/mL of IL-2 throughout.

2.9. Incubate at 37 °C. Change 50% of the media using TCM with 300 IU/mL IL-2 but without PHA-L on days 5−7.

3. Expansion of Treg and maintenance of clones in culture

3.1. Beginning on day 12, check cultures for the presence of pellets of proliferating cells.

NOTE: There will be pellets formed by feeder cells, but on microscopic examination those will appear as small, round, dying or dead cells, whereas proliferating T cells will be larger in size, with bright contrast and healthy appearance, often forming clusters of adjacent cells that appear brown on macroscopic examination.

3.2. Continue to examine cultures every 1−2 days. Isolate proliferating tentative clones by transferring them onto single wells. Place each tentative clone on a single 96 well plate to avoid cross-contamination of any given tentative or established clone though inadvertent cell transfer from other wells.

3.3. Maintain cells through 50% media changes every 2−3 days and split as necessary. Monitor cells closely.

NOTE: Delaying media changes and splitting can harm cells and “over splitting” can arrest their proliferation and result in cell death.

3.4. Restimulate cells with irradiated allogeneic feeders as described in steps 2.3−2.9, but use lower PHA-L concentrations (i.e., 1 µg/mL in TCM with 300 IU/mL IL-2). Restimulate when cell size decreases and cells become round as opposed to elongated oval which is typically after 2−3 weeks.

NOTE: Maintain the original cloning culture for at least 6−8 weeks. Many of the ‘early’ clones that become visible at or shortly after 2 weeks tend to not be true Treg, but fast proliferating non-Treg/Teff cells and are more likely to fail vetting than ‘late’ cells, some of which will take more than 1 month to visibly appear in culture.

4. Vetting of tentative Treg clones

4.1. Begin vetting of tentative clones once they have proliferated to ~1 x 10^6 cells for monoclonality and TSDR methylation status.

4.2. Optionally, prescreen cell expansions by staining for CD3, CD4, CD127, and CD25 in order to identify those of interest (i.e., $CD3^{+}CD4^{+}CD127^{lo}CD25^{hi}$) for further assessment (**Figure 2**).

4.3. Establish monoclonality through identification of the presence of a single Vβ chain in the cellular product by Vβ staining using sets of commercially available staining antibodies (**Table of Materials**; **Figure 3**) following manufacturer's instructions. Ensure to acquire at least 1 x 10^6 events when running samples on a flow cytometer for a reliable analysis, so that minor contaminating populations can be detected. Use a viability dye.

4.4. Have TSDR methylation status at the *FOXP3* locus assessed by commercial providers or in-house[11,12]. Obtain at least 1 x 10^5 cells for adequate results.

4.5. Once Treg identity and clonality have been confirmed in steps 4.3 and 4.4, cryopreserve the cells or use for downstream applications.

NOTE: Alternative, but far less reliable vetting approaches, are determining Treg phenotype by FACS stainings[1,10] and in vitro or in vivo suppression assays[13,14]. Avoid overreliance on Treg suppression assays[13]. The assays are typically, but not exclusively, based on the coculture of Treg in graded numbers with responder T cells (i.e., PBMC or purified T cells, sometimes Treg-depleted) in the presence of third party antigen-presenting cells or CD3/CD28 with dye-dilution representing proliferation as the read-out. Many of these assays have severe limitations and may either over- or underestimate the actual degree of suppression mediated by Treg. TSDR methylation status remains the most definite/reliable measure of the Treg phenotype or "identity"[3,15,16]. Note that FOXP3 is located on the X chromosome and, in females, one X chromosome is inactivated by DNA methylation, which affects the results of TSDR methylation analysis.

5. Cryopreservation of Treg

NOTE: Treg can be stored long-term after successful cryopreservation with DMSO and PHS-AB.

5.1. Determine the number of cryovials required to cryopreserve the cells in. This is equal to the total final volume of cell suspension (in mL) to be cryopreserved, because 1 mL is frozen per vial. Add a 10% safety margin to this volume in order to account for losses due to pipetting/surface tension.

NOTE: Cells can be cryopreserved at a wide range of concentrations, typically in between 0.1−100 x 10^6 cells/mL.

5.2. Label the cryovials.

5.3. Generate solution A (SA): mix 50% RPMI and 50% PHS-AB or human plasma. If plasma is used, spin at 2,000 x *g* for 20 min at 4 °C.

NOTE: The volume of SA should be 75% of the total final volume of cell suspension to be cryopreserved as determined in step 5.1.

5.4. Generate solution B (SB): mix 60% (v/v) PBS or RPMI and 40% (v/v) dimethyl sulfoxide (DMSO).

NOTE: The volume of SB should be 25% of the total final volume of cell suspension to be cryopreserved as determined in step 5.1.

5.5. Chill both solutions to 4 °C.

5.6. Prepare the freezing medium by mixing all of SB with equal parts of SA and keep on ice.

5.7. Spin Treg from step 4.5 at 450 x *g* for 5 min at 4 °C. Aspirate and discard media, and resuspend cells in the remaining ice-cold SA and keep on ice.

5.8. Slowly add the chilled freezing medium 1:1 to the cells resuspended in SA in a large tube on ice while shaking the tube.

5.9. Quickly aliquot into cryovials at 1 mL/vial. Immediately place the cryovials into a cupboard box at RT and transfer to a -80 °C freezer without delay. Transfer to liquid N_2 after 24 h.

REPRESENTATIVE RESULTS:

Successful implementation of this protocol will lead to the generation of stable human regulatory T cell clones and lines.

Preselection/preenrichment of $CD4^+CD127^{lo}CD25^{hi}$ cells was a straightforward method to obtain a starting population that contained most human Treg (**Figure 1A–C**). Not all clones displayed a Treg phenotype. Prescreening of clones by measurement of $CD25^{hi}CD127^{lo}$ expression was a reasonable approach to identify clones of interest, and increased efficiency of generating target clones through their preselection (**Figure 2A**,**B**). Assessment of clonality could be achieved easily by staining for TCR Vβ, and required the demonstration of one single Vβ chain in the cellular product (**Figure 3A**,**B**). Characterization of clones solely using flow cytometry may lead to misinterpretation of results, however, due to imperfect separation of Teff and Treg cells, in particular as shown for FOXP3, CD25, and CD127 stainings (**Figure 4A**). The combined use of CD45RA and FOXP3/CD25 may provide better clues, but the critically important differentiation between intermediate and high FOXP3, and/or CD25 in the CD45RA negative subset ($FOXP3^{int}$ $CD45RA^{neg}$ cells do not suppress) can be challenging, because fluorescent minus one (FMO) gating controls are of limited help in this scenario, biological controls (ideally Treg clones as in this example) may not be available initially, and stainings of native “control” PBMC/T cells can be difficult to optimize and/or interpret[10]. While combined stainings of FOXP3 and HELIOS typically gave the most reliable results, the use of HELIOS as a Treg marker remains somewhat

controversial, as opposed to methylation status at the TSDR, which is widely accepted. **Figure 4** shows a comparison of one Teff and one Treg clone by flow cytometry (**Figure 4A**) and by single cell RNA sequencing (**Figure 4B**) with VD(J) analysis, demonstrating nonoverlapping phenotypic and gene expression profiles. Their TCR repertoire diversity was 1 in both cell products (i.e., only one Vβ chain is expressed through a unique VD(J) recombination), providing evidence of monoclonality. In **Figure 5**, the kinetics of a Treg cell clone proliferation cycle was evaluated by Ki67 staining over time, demonstrating the slow proliferation of most genuine Treg clones.

FIGURE AND TABLE LEGENDS:

Figure 1: Preenrichment of a Treg-containing cell population. (**A**) Human $CD3^+CD4^+$ $CD127^{lo}$ $CD25^+$ cells within PBMC gated on singlet live $CD3^+CD4^+$ lymphocytes. This population contained most human regulatory T cells and served as the starting population for enrichment. (**B**) Enrichment for $CD127^-CD4^+$ T cells through negative selection using magnetic sorting. (**C**) Further enrichment of $CD25^{hi}$ cells (red, histogram) from the $CD127^-CD4^+$ population (blue, histogram) could be achieved either though magnetic (as shown here) or FACS sorting. Green represents the CD25 expression in the PBMC population prior to enrichment.

Figure 2: Screening of cell expansions for *bona fide* Treg. Flow cytometric assessment of CD127 and CD25 expression levels was a straightforward approach for initial screening, but required confirmation of methylation status at the TSDR given the high number of "false" (i.e., methylated) samples. (**A**) Cell expansions obtained 14 days after cloning: only 1 (6%) of cell expansions showed demethylation at the TSDR consistent with *bona fide* Treg. (**B**) Cell expansions detected after 30 days: 2 (33%) out of 6 expansions were consistent with Treg per methylations status. Gated on live cells. Cells are $CD4^+CD3^+CD8^-$.

Figure 3: Verification of clonality. Monoclonality of the cell expansions must be verified. (**A**) Clones were stained positive for one Vβ stain; in the case displayed, $V\beta_{17}$. (**B**) Shows the entire data set for 24 human Vβ specificities. (**C**) Principle of the assay. Cells were stained with FITC/PE single or double conjugated antibodies, each specific for one Vβ chain of the human T cell receptor. Each quadrant above threshold displayed staining for one single Vβ chain, but not others.

Figure 4: Phenotypic assessment of Treg clones after long-term expansion. (**A**) Flow cytometric analysis of Treg (red) and Teff (blue) clone in overlay plots demonstrates that differentiation between Treg and Teff can be difficult given the low levels of FOXP3 and CD25 expression in human Teff. (**B**) Single cell RNA sequencing with V(D)J analysis of the same cellular products as in panel A demonstrates the high differential expression of HELIOS, FOXP3, and CD25 in the clonal Treg vs. clonal Teff, and the absence of IFNg and CD127 expression after 51 weeks in culture. The TCR diversity in each sample is 1 (i.e., expansions are monoclonal).

Figure 5: Proliferation of Treg in culture. A typical expansion cycle in feeder-stimulated culture lasts for about 2 weeks and then necessitates restimulation. Overlay plot of Ki67 expression of a Treg clone, at 7 (red), 11 (blue), and 14 (green) days is shown. Dying feeder cells were excluded

through viability staining and gating on HLA-A24 which was expressed by the Treg, but not the feeder cell donor.

Table 1: Troubleshooting.

DISCUSSION:

This protocol describes the propagation of ultrapure human regulatory T cells through the isolation, expansion, and careful vetting of cells obtained in a limiting dilution and feeder cell-based expansion approach from Treg-containing starting populations.

Critical steps in this approach are: 1) the choice of an appropriate starting population. Generally, the $CD127^{lo}CD25^{hi}$ compartment of $CD4^+$ T cells within human PBMC contains a wide variety of Treg that suits this purpose[10,17]. 2) Accurate cell counts are mandatory for successful cloning given the risk of either seeding too many cells, which can subsequently generate oligoclonal cells lines, or too seeding too few, reducing cloning efficiency. This protocol uses a standard hemocytometer for counting. Automated cell counters, if settings are properly optimized and counts are vetted, may be used alternatively. 3) Long-term observation and maintenance of plates with seeded primary cells. Treg proliferate slowly, which means that clones may become macroscopically visible in the form of cell pellets only after several weeks in culture. It is important to note that this time frame differs significantly from that typical for similar cloning approaches applied in the generation of fast proliferating Teff, such as cytotoxic $CD8^+$ T cells or others. 4) Critical assessment of slowly proliferating clones. The slow proliferation rate of Treg often creates problems not usually faced in irradiated feeder cell cloning approaches, where feeder cells typically die several days after irradiation in coculture with rapidly proliferating clones or cell lines. Given the low expansion rates of Treg, feeder cells often retain some viability in culture for more than 1–2 weeks, despite irradiation. This can affect the interpretation of results from the analysis of the resultant cellular product and mislead the investigator. It is therefore recommended to sort out feeder cells that may have retained some viability on grounds of HLA expression, which should be different from that of the expanded Treg. All feeders die ultimately if properly irradiated. 5) Vetting of clones by pyrosequencing to date is the most reliable method of establishing the presence of *bona fide* Treg in the culture. Treg suppression assays and phenotyping all have severe limitations that do not always allow a reliable assessment. Pyrosequencing can be outsourced to commercial providers or done in-house. To limit time and costs spent on vetting cells, it is reasonable to first establish monoclonality, followed by phenotypical screening (e.g., CD127/CD25 expression) eliminating clones with non-$CD127^{lo}CD25^{high}$ phenotypes (although this may exclude some *bona fide* Treg) and then proceed to pyrosequencing with the selected clones.

Table 1 provides a tool for fast troubleshooting.

[Place Table 1 here]

Possible modifications of this method include: 1) Treg isolation method. Use of FACS is possible, but subject to the disadvantages mentioned above. It does, however, allow better immediate control of the phenotypic properties of the starting population targeted and can be preferable if a specific starting population of interest is not easily obtained by magnetic sorting. 2) Choosing a specific Treg subset as the starting population. For example, TIGIT/CD226 vs. $CD39^{+}$ Treg vs. standard or $CD45RA^{+}FOXP3^{int}$ vs. $CD45RA^{-}FOXP3^{hi}$. Several phenotypic characteristics have been proposed to demarcate Treg subsets especially amenable to stable expansion and high suppressive activity[18,19]. 3) Cryopreserved vs. fresh feeder cells. The use of thawed (cryopreserved irradiated or non-irradiated with subsequent irradiation) feeder cells may be possible but is not recommended and has not been systematically evaluated for this protocol. 4) Vetting of clonality by PCR vs. flow cytometry. Sequencing offers better coverage than TCR Vβ staining and may be preferable for determination of monoclonality[20], but requires more effort and time to set up than Vβ staining. 5) In vivo suppression assays such as xenogeneic GVHD models in NSG mice. These may be preferable to in vitro assays but are time-consuming, labor-intensive, and expensive[14]. They may be feasible in settings where colonies of NSG mice or other appropriate immune-incompetent murine recipients of human cells are routinely maintained. 6) Generation of antigen-specific Treg clones. This can be attempted through the application of an appropriate approach for the isolation of antigen-specific Treg, such as tetramer/polymer stainings, responder cell isolation in antigen (AG)-specific stimulation assays, or other similar technique, and the subsequent application of this protocol. However, tetramer staining may alter the viability of isolated cells and more cells may have to be seeded.

Limitations of this protocol include: 1) Slow proliferation of Treg limits cell yield. Slow proliferation of Treg is the main limitation to their long-term in vitro expansion. This applies to the protocol presented here. 2) Unknown antigen specificity. The current protocol does not select Treg based on known AG specificity. While the monoclonality of the resultant product implies a high degree of specificity to AG, the specificity is not known *a priori*. Choosing a starting population with known AG specificity may be challenging given the low precursor frequency of AG-specific Treg in human peripheral blood, but can be tackled by preenrichment of Treg previously expanded in response to the desired antigen(s), direct targeting using polymeric MHC-peptide approaches, or other methods. 3) Use of human material in the expansion process may limit therapeutic applications. The use of human feeder cells poses a challenge for therapeutic applications but does not preclude it. All reagents necessary to carry this protocol out are available in Good Manufacturing Practice (GMP) standards (IL-2 and PHA-L). The use of artificial antigen-presenting cells (APC) and cytokine cocktails might help alleviate the need for feeder cells, but remains to be tested. 4) Cells are long-term, in vitro expanded. This means that there is no guarantee that these cells adequately reflect physiologically occurring Treg given their long-term in vitro culture.

The main significance of this approach versus existing/alternative methods lies in its capacity to generate an ultrapure Treg cell product that maintains Treg-defining specifications over very long periods of time in culture. The cloning approach allows the investigator to control the properties of the cells allowed to move on in expansion experiments, therefore eliminating the propagation

of Teff-type cells. Most other methods have relied on the polyclonal or oligoclonal expansion of Treg, which often leads to compromised suppressor function over time.

Future applications and directions of the method include: 1) Generation of AG-specific Treg clones. This may partially circumvent the limitations related to the slow proliferation of *bona fide* Treg and reduce the need for large numbers of Treg in targeted clinical applications. 2) Applications where low numbers of Treg are required. This includes phenotypic and functional analysis of the clones (e.g., RNA sequencing). 3) Downstream gene-editing approaches. This method can be combined with CRISPR-Cas9 modification, for example, to allow the assessment and manipulation of specific gene functions in human Treg.

ACKNOWLEDGMENTS:

This project was supported by the National Eye Institute of the National Institutes of Health under Award Number K08EY025324 (Nowatzky) and by a Colton Scholar Award from the Judith and Stewart Colton Center for Autoimmunity (Nowatzky).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES :

1. Miyara, M. et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity.* **30** (6), 899-911 (2009).
2. Polansky, J. K. et al. DNA methylation controls Foxp3 gene expression. *European Journal of Immunology.* **38** (6), 1654-1663 (2008).
3. Toker, A. et al. Active demethylation of the Foxp3 locus leads to the generation of stable regulatory T cells within the thymus. *The Journal of Immunology.* **190** (7), 3180-3188 (2013).
4. Garg, G. et al. Blimp1 Prevents Methylation of Foxp3 and Loss of Regulatory T Cell Identity at Sites of Inflammation. *Cell Reports.* **26** (7), 1854-1868 e1855 (2019).
5. Brunstein, C. G. et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood.* **117** (3), 1061-1070 (2011).
6. Hippen, K. L. et al. Massive ex vivo expansion of human natural regulatory T cells (T(regs)) with minimal loss of in vivo functional activity. *Science Translational Medicine.* **3** (83), 83ra41 (2011).
7. Bluestone, J. A. et al. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Science Translational Medicine.* **7** (315), 315ra189 (2015).
8. Dromey, J. A. et al. Generation and expansion of regulatory human CD4(+) T-cell clones specific for pancreatic islet autoantigens. *Journal of Autoimmunity.* **36** (1), 47-55 (2011).
9. Sabado, R. L. et al. Preparation of tumor antigen-loaded mature dendritic cells for immunotherapy. *Journal of Visual Experiments.* (78), e50085 (2013).
10. Nowatzky, J., Stagnar, C., Manches, O. OMIP-053: Identification, Classification, and Isolation of Major FoxP3 Expressing Human CD4(+) Treg Subsets. *Cytometry A.* **95** (3), 264-267 (2019).
11. Zhang, Y. et al. Genome-wide DNA methylation analysis identifies hypomethylated genes regulated by FOXP3 in human regulatory T cells. *Blood.* **122** (16), 2823-2836 (2013).

12. Spreafico, R. et al. A sensitive protocol for FOXP3 epigenetic analysis in scarce human samples. *European Journal of Immunology.* **44** (10), 3141-3143 (2014).
13. Collison, L. W., Vignali, D. A. In vitro Treg suppression assays. *Methods in Molecular Biology.* **707** 21-37 (2011).
14. Workman, C. J. et al. In vivo Treg suppression assays. *Methods in Molecular Biology.* **707** 119-156 (2011).
15. Feng, Y. et al. Control of the inheritance of regulatory T cell identity by a cis element in the Foxp3 locus. *Cell.* **158** (4), 749-763 (2014).
16. Toker, A., Huehn, J. To be or not to be a Treg cell: lineage decisions controlled by epigenetic mechanisms. *Science Signaling.* **4** (158), pe4 (2011).
17. Liu, W. et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *Journal of Experimental Medicine.* **203** (7), 1701-1711 (2006).
18. Fuhrman, C. A. et al. Divergent Phenotypes of Human Regulatory T Cells Expressing the Receptors TIGIT and CD226. *The Journal of Immunology.* **195** (1), 145-155 (2015).
19. Gu, J. et al. Human CD39(hi) regulatory T cells present stronger stability and function under inflammatory conditions. *Cellular & Molecular Immunology.* **14** (6), 521-528 (2017).
20. Genevee, C. et al. An experimentally validated panel of subfamily-specific oligonucleotide primers (V alpha 1-w29/V beta 1-w24) for the study of human T cell receptor variable V gene segment usage by polymerase chain reaction. *European Journal of Immunology.* **22** (5), 1261-1269 (1992).

Figure 1

Click here to access/download;Figure;FIg 1_R.psd

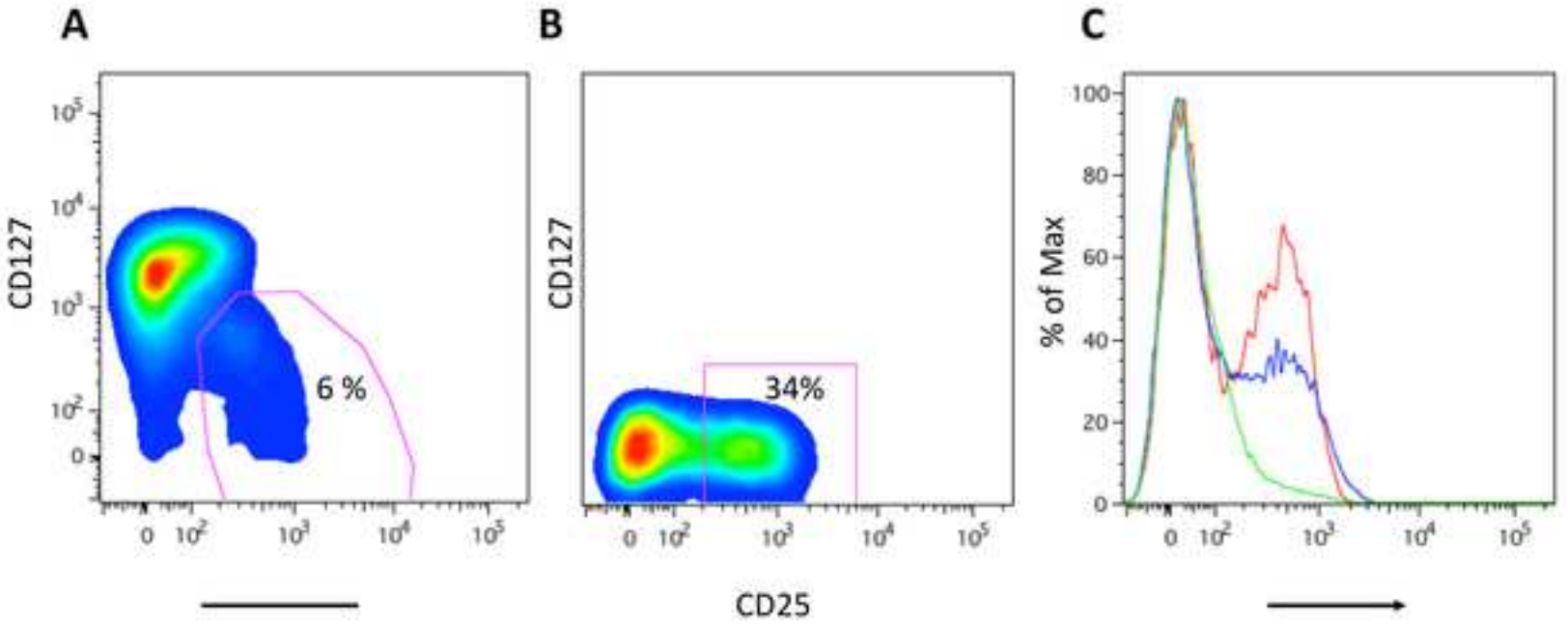


Figure 2

Click here to access/download;Figure;Fig. 2_R.psd

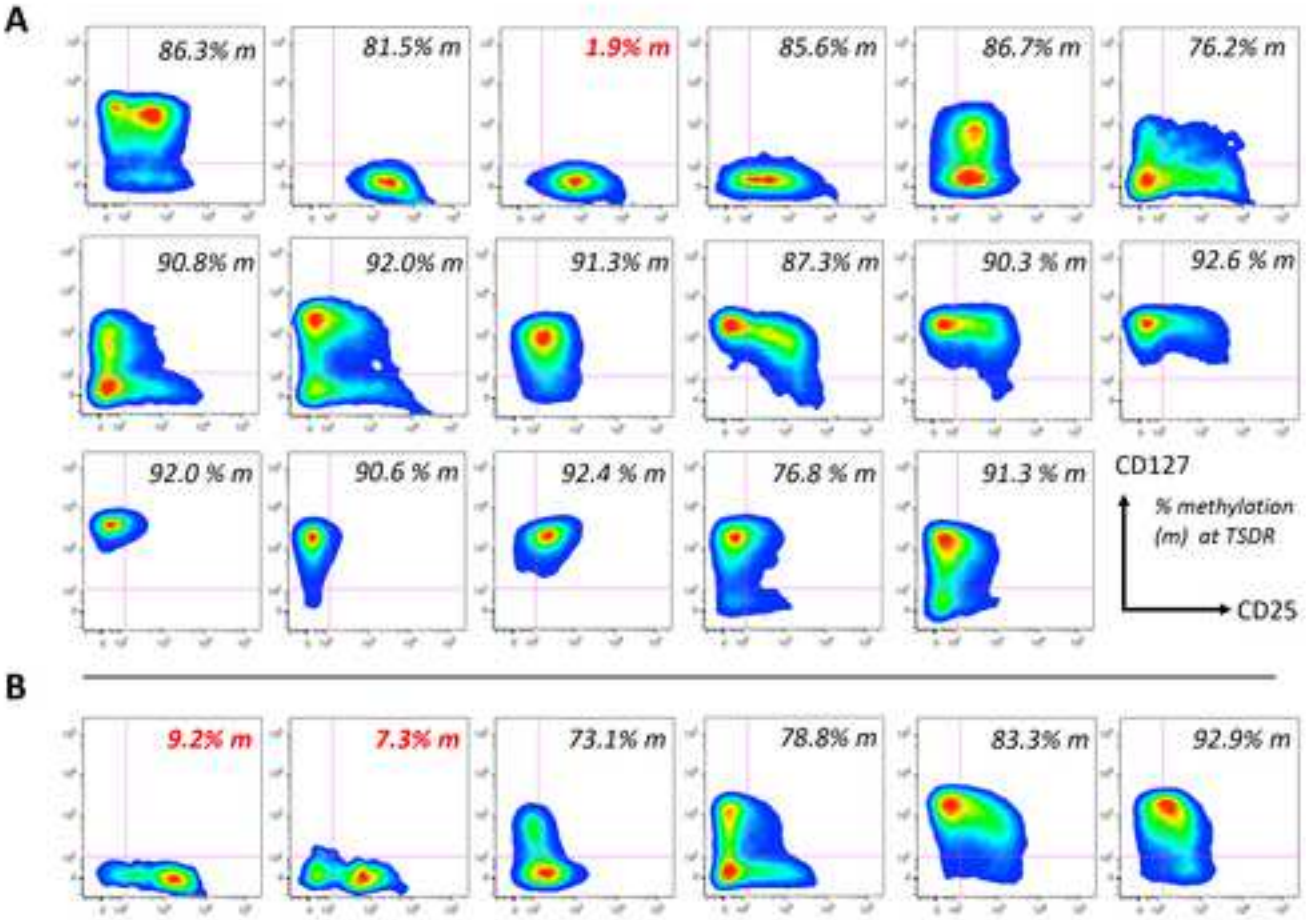


Figure 3

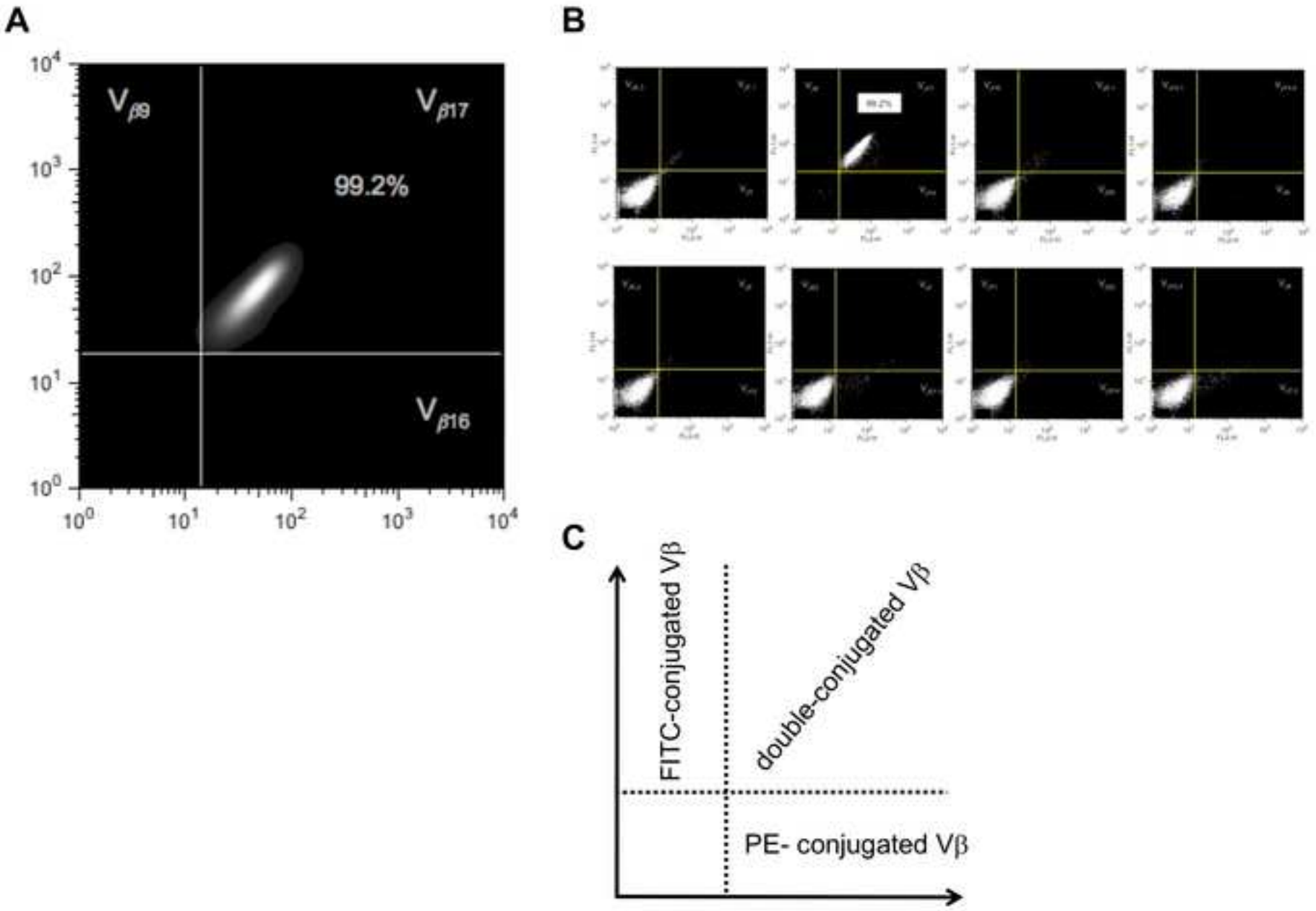


Figure 4

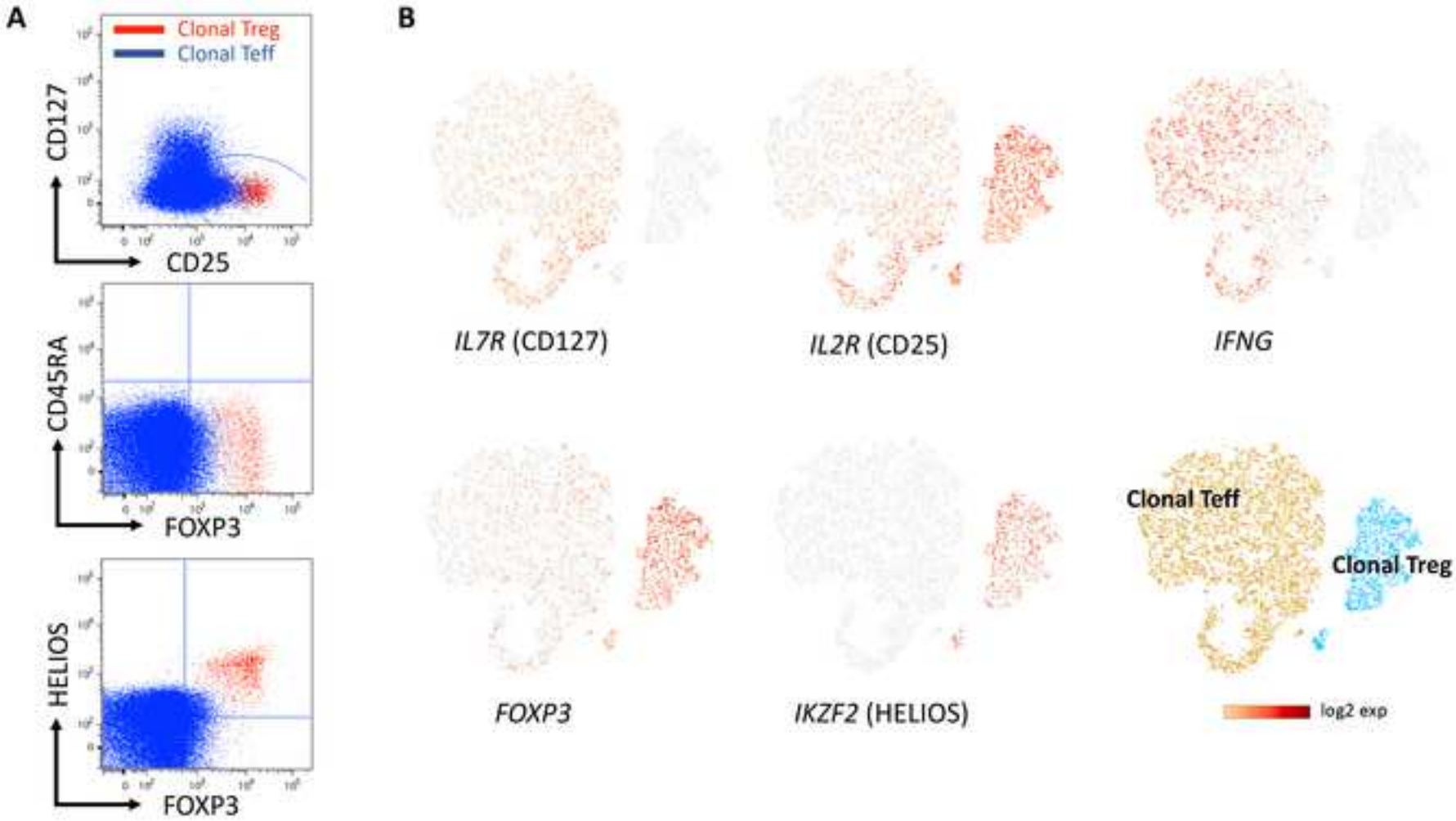
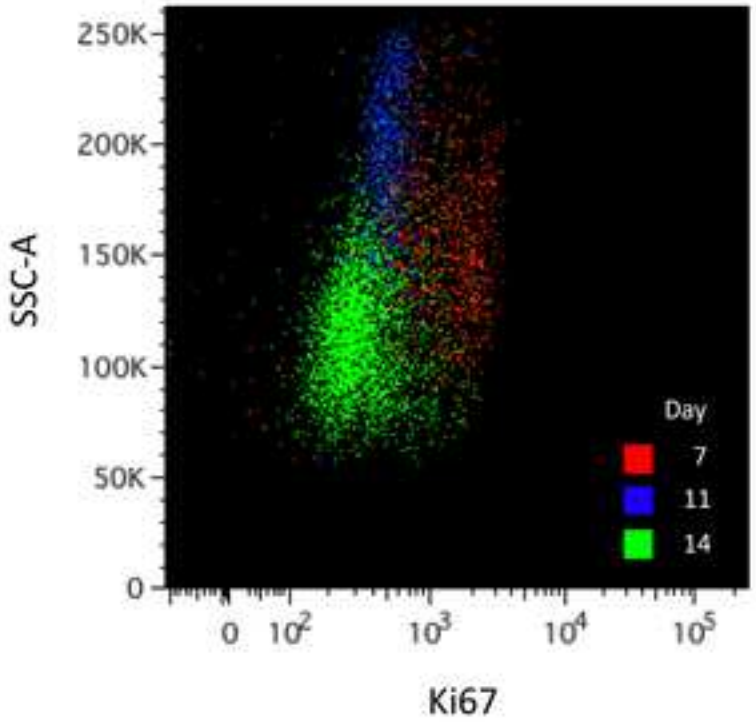


Figure 5

Click here to access/download;Figure;Fig 5_R.psd



Problem
No clones are seen after 2 weeks.
Clones obtained do not show a Treg phenotype on flow cytometry analysis.
Cell lines obtained are not clonal.
Clones show Treg phenotypes by flow cytometry and suppress in in vitro suppression assays by are not demethylated at the TSDR.
Treg clones with acceptable initial proliferation cease to expand in subsequent expansion/stimulation cycles.
Viable feeder cells are detectable by flow cytometry when analyzing the cell line/clone.

Possible explanation and approach
Wait and continue to maintain the culture. Treg expand slowly and typically need more time to form visible cell pellets than Teff.
1) *Clones are Teff.* Harvest clones that become visible later—they are more likely to be Treg, given slow proliferation. 2) *Staining panel is insufficiently optimized.* Refine panel or use a published optimized Treg staining panel[10]. Verify phenotypically promising clones through pyro-sequencing at the TSDR.
Make sure cells are counted accurately when seeded and have not been allowed to sediment prior to seeding. Add PHA-L immediately prior to seeding when plating feeders and primary Treg together, or plate out Treg first and then add PHA and feeders. PHA causes clumping of cells and can negatively impact on maintaining a single cell suspension when plating Treg enriched cells. Make sure feeder cells are dead at the time of analysis or sorted out if in doubt (see comments on extended survival of irradiated feeders when co-cultured with slowly-expanding primary cells).
Demethylation at the TSDR supersedes suppression assays and flow cytometry analysis results in determination of Treg identity. Effector cells can reduce cell numbers of the responder T cell population in a dose-dependent manner through cytotoxic and other effects. Flow cytometry results can be difficult to interpret for clones when accurate biologic controls cannot be obtained given variable levels of FoxP3 and CD25 expression in Treg and Teff.
Try to complete the experiment—some Treg clones will stop expanding. Attempt expansion with different allogeneic feeder cell donors—some work better than others. If cells previously proliferated well with a specific feeder cell donor, it can be helpful to expand using cells from the same donor again in subsequent expansion cycles if feasible.
This may occur when Treg proliferate slowly as there is less competition allowing irradiated feeder cells to survive longer than usual. Sort out clones based on HLA expression that is present in on the Treg, but absent on the feeder cells. Make sure to use a viability dye. All properly irradiated feeders will ultimately die.

Name of Material/Equipment	Company	Catalog Number
0.22 µm Stericup, 500 mL	Millipore	5500
100x Nonessential amino acids	Gibco	11140-050
15 mL conical centrifuge tubes (50/bag, case of 500)	ThermoFisher Scientific	339650
1M HEPES	Gibco	15630-080
25 ml Single Well Pipet Basin	Fischer Scientific	13-681-508
50 mL Conical Centrifuge Tube (25/sleeve)	ThermoFisher Scientific	339652
50x Penicillin Streptomycin Soln	Corning	Corning, 30-001-Cl
CryoTube Vial Int Thread Round Btm Starfoot PP Screw Stopper Sterile PP 1.8 mL	Nalge Nunc	377267
DMSO	Corning	25-950-CQC
EasySep Human CD25 positive selection kit	Stemcell Technologies	18231
EasySep Human CD4+CD127low T cell Pre-Enrichment Kit	Stemcell Technologies	19231
EasySep Human CD4+CD127lowCD25+ Regulatory T Cell Isolation Kit (alternative to item 12)	Stemcell Technologies	18063
Ficoll	GE Healthcare	17-5442-03
Human AB Serum (PHS-AB)	Valley Biomedical Inc	HP1022
LIVE/DEAD Fixable Blue Dead Cell Stain Kit, for UV excitation	Thermo Fischer	L-34962
Phytohemagglutinin-L (PHA-L)	Millipore/Sigma	11249738001
Recombinant IL-2 (e.g., PROLEUKIN[â])	Prometheus	
RPMI 1640	Gibco	21870-076

Staining antibodies for flowcytometry (Treg phenotyping)	See "Comments"	See "Comments"
TCR Vβ Repertoire Kit; IOTest Beta Mark	Beckman Coulter	PN IM3497
Tissue Culture Plate, 96 Well, U-Bottom with Low Evaporation Lid	Corning	353077

Comments/Description

Media storage and preparation

Media component

Media component

Media component

Alternatives are FACS or MACS column-based sorting

Alternatives are FACS or MACS column-based sorting

Alternatives are FACS or MACS column-based sorting

PBMC purification from peripheral blood of leukapheresis products; density gradient medium

Media component

Viability dye

T cell stimulation

T cell stimulation and maintenance/ Media component

Media component

Staining antibodies are enlisted in: Nowatzky et al. (2019) PubMed PMID: 30584695; PubMed Central PMCID: PMC6497402. In case EasySep Human CD25 positive selection kit is used, stain with 2A3 or BC96 anti-CD25 antibody, e.g.: Brilliant Violet 421 anti-human CD25 Antibody (Biolegend; 302629)

Vetting of expansions for monoclonality

Editorial comments:

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.

Thank you very much for your time and work. We have retained the journal's formatting style. All changes – including the markings of the parts suggested for filming – are contained in it.

2. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We have tried to address this. Please refer to the revised manuscript.

3. Please address specific comments marked in the attached manuscript.

This has been done. Please refer to the revised manuscript.

4. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

This has been done. Please refer to the revised manuscript.

5. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

This has been done. Please refer to the revised manuscript.

6. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

This has been done. Please refer to the revised Table of Materials.