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Standardization, Evaluation, and Area-Under-Curve Analysis of Human and Murine Treg Suppressive Function

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Abstract

FOXP3+ T-regulatory (Treg) cells have important roles in immune homeostasis, and alterations in their number and function can predispose to diseases ranging from autoimmunity to allograft rejection and tumor growth. Reliable identification of human Tregs remains a persistent problem due to a lack of specific markers. The most definitive Treg characterization currently involves combined assessment of phenotypic, epigenetic and functional parameters, with the latter typically involving in vitro Treg suppression assays. Unfortunately, suppression assays are frequently performed using differing methods and readouts, limiting comparisons between studies. We provide a perspective on our experience with human and murine Treg suppression assay conditions, including Treg data obtained in clinical transplant studies, Tregs isolated from healthy donors and treated with epigenetically active compounds, and Tregs from standard murine strains (C57BL/6 and BALB/c). We provide detailed descriptions and illustrations of typical problems, shortcomings and troubleshooting; describe new modifications and approaches; and present a new method for calculation of suppressive assay data using a modified area-under-curve (AUC) method. This method allows us to directly compare Treg suppressive function between multiple patients (such as in clinical transplant studies), to reliably track changes in Treg function from the same person over time, or compare effects of Treg-modulating compounds tested with different healthy donors Tregs in separate or combined experimental settings.

Keywords

FOXP3+ regulatory T cells; Tregs; Suppression assay

1 Introduction

FOXP3+ T-regulatory (Treg) cells comprise a small subset of CD4+ T cells with bystander suppressor activity. Since their initial characterization as CD4+ CD25+ T cells by Sakaguchi and colleagues in 1995 [1], Tregs have been a focus of interest for the development of new diagnostic and therapeutic strategies for immunologically mediated diseases. FOXP3 is a key marker for use in the identification of murine Tregs [2], and the development of YFP/GFP- Foxp3 knock-in mice facilitated isolation of live murine Tregs for in vitro and in vivo functional studies [3]. However, in addition to Tregs, activated human effector T (Teffs) cells can upregulate FOXP3 [4], such that phenotypic evaluation of human Tregs using FOXP3 alone is not sufficiently specific for clinical studies. To overcome this problem, various

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additional Treg-associated markers with supposed preferential expression by FOXP3+ cells have been proposed, but in most cases direct validation of their Treg specificity in human clinical samples was not shown. Indeed, FOXP3 is an indispensable, but not sufficient marker of Treg cell fate. Thus, to maintain their Treg function and phenotype, an additional set of FOXP3-independent genes, including *CTLA4* and *IKZF2*, need to be demethylated [5]. Likewise, a quintet of transcription factors, namely IRF4, Eos, Gata1, Satb1, and FOXP3, but not FOXP3 alone, can reproduce Treg-like gene expression in Teff cells [6]. As a result, a “gold standard” for reliable phenotypic Treg evaluation is still lacking, and functional studies remain the most reliable way to confirm that CD4+CD25+FOXP3+ cells under study are indeed Treg cells.

The first in vitro suppression assays were described over a decade ago [7, 8]. Since then, many modifications of activation and co-stimulation conditions, time in culture, methods for readout of T cell proliferation, and calculations of results were developed. These modifications have led to controversy due, in part, to poor reproducibility between different research groups. An “ideal” Treg suppression assay would be sensitive enough to show alteration in suppressive function, and, at the same time, should not be prone to generate misleading results due to small variations in experimental conditions such as different lots of sera, stimulation reagents, etc., altered resistance of Teffs to Treg suppression, or differing characteristics of antigen-presenting cells (APC). For this purpose, each key assay component should be standardized, have minimal assay-to-assay variability, and provide the researcher with data for standardization controls in each experiment.

In this chapter, we discuss various technical considerations that must be taken into account especially when using human Treg suppression assays, since these more challenging and difficult to normalize than murine Treg suppression assays, as well as additional features of Tregs we have noted in ongoing clinical studies. We also discuss the most common pitfalls and drawbacks of this method, and illustrate issues using our data accumulated over 6 years of work with human Tregs, and a decade of work with murine Tregs. Finally, we suggest a new sensitive and reproducible method for calculation of Treg suppressive function that can be used to compare experimental data.

2 Materials

1. Ficoll-Paque PLUS Medium (GE Healthcare).
2. ACCUSPIN™ Tubes Sterile 50 mL (Sigma) or SepMate 50 mL tubes (Stemcell Technologies), or BD Vacutainer CPT tubes, 8 mL.
3. Ca^{2+} and Mg^{2+} -free DPBS.
4. $10\times$ Ca^{2+} and Mg^{2+} -free DPBS or red blood cell lysis buffer.
5. Heat-inactivated fetal bovine serum (FBS).
6. Trypan blue (Sigma).
7. For murine cells and human tissue samples: Cell Strainers 70 μm or Nylon Mesh 70 μm .

8. Cell isolation buffer: DPBS supplemented with 2 mM EDTA and 0.5 % FBS.
9. For human cells: CD4+CD25+ Regulatory T cell isolation kit, human (Miltenyi) and for murine cells: CD4+CD25+ Regulatory T Cell Isolation Kit, mouse (Miltenyi).
10. For human cells: CD3 MicroBeads, human (Miltenyi) and for murine cells: CD90.2 MicroBeads, mouse (Miltenyi). Both reagents are optional.
11. LD columns and LS columns (Miltenyi).
12. T-cell media: RPMI1640 (Invitrogen), supplemented with 10 % heat-inactivated FBS, penicillin and streptomycin, and 2-mercaptoethanol (10 0 µM).
13. CFSE or CellTrace (Invitrogen).
14. For human cells: MACS GMP CD3 antibody (Miltenyi) and Dynabeads® M-450 Tosylactivated beads (Invitrogen). For murine cells: anti-mouse CD3 functional grade purified antibody.
15. 96-Well U-bottom plates or 96-well V-bottom plates for low cell numbers.
16. (Optional) CryoStor® CS5 cell cryopreservation media (Sigma) and cryovials 1–2 mL.
17. Flow cytometry buffer: DPBS supplemented with 2 % heat-inactivated FBS.
18. Fixable LIVE/DEAD (Invitrogen) or fixable Zombie (Biolegend) or fixable Viability Dye (eBioscience).
19. Transcription Factor Buffer Set (BD Biosciences) or Foxp3/ Transcription Factor Staining Buffer Set (eBioscience).
20. Anti-human or anti-mouse CD4, FOXP3, CD8 (optional), CD25 (optional), CD127 (optional) and CTLA4 (optional) antibodies for evaluation of human or murine cells, correspondingly. Ki- 67 antibody (optional) from BD Biosciences works for both species.

3 Methods

3.1 Human PBMC Isolation

For blood samples that were collected the same day, use ACCUSPIN, SepMate or CPT tubes according to corresponding manufacturer's instruction. Fill ACCUSPIN or SepMate with Ficoll-Paque PLUS. You may also isolate PBMC using standard sterile 50 or 15 mL tubes, according to protocol in Ficoll-Paque PLUS package.

If blood has to be kept overnight or was shipped overnight («old» blood), then maintain the room temperature of blood until PBMC is isolated, and follow this modified procedure:

1. Warm, and then keep all reagents for PBMC isolation from «old» blood, at room temperature. It is also important to set up centrifuge for room temperature. Cold may provoke massive hemolysis of «old» erythrocytes which decreases PBMC yield and quality.

2. Evaluate blood volume. Use ACCUSPIN tubes for 15–21 mL of undiluted «old» blood, or SepMate for 16–26 mL of 2×-diluted blood. Dilute blood with DPBS + 2 % FBS. Combine both types of tubes if needed. Alternatively, you may use 2×-diluted blood and standard sterile 50 mL or 15 mL plastic tubes, according to protocol in Ficoll-Paque PLUS package, or combine all types of tubes.
3. Fill ACCUSPIN (by centrifugation at $800 \times g$, 30 s) and SepMate (by pipetting) tubes with room-temperature Ficoll-Paque PLUS, 15–15.5 mL in each 50 mL tube. Avoid air bubbles under the membranes.
4. Add corresponding volume of blood and spin down tubes at room temperature, $400 \times g$, 35 min. Higher speed with «old» blood samples may destroy red blood cells and lead to massive hemolysis. Make sure that centrifuge is set up as «no brake» (no rapid deceleration).
5. Remove plasma layer and collect PBMC cells within mononuclear band.
6. Wash PBMC with room temperature DPBS, 10 min at $300 \times g$.
7. Remove supernatant and evaluate whether red blood cell lysis is required. If not, continue with **step 9**.
8. Proceed with erythrocytes lysis if needed. Human PBMC sustain well the hypotonic shock. For that, tap tube to loosen cell pellet, add 18 mL of sterile DI water, mix for 5–10 s, and add 2 mL of $10\times$ Ca^{2+} and Mg^{2+} -free DPBS. Mix, add sterile DPBS to 50 mL, and wash for 10 min at $300 \times g$.
9. Resuspend cells in cell isolation buffer, and evaluate cell numbers and viability using Trypan blue staining.

3.2 Human Tissue Cell Isolation and Murine Cell Isolation

1. Obtain spleen, peripheral, and mesenteric lymph nodes of sacrificed mice and collect them into tubes with 4 °C sterile DPBS. Obtain human lymph nodes, collected into tubes with 4 °C cell culture media.
2. Use a plunger of a 5 mL syringe and cell strainer or mesh cuts to prepare single-cell suspension in Petri dishes. Collect cells into 50 mL tubes with inserted cell strainer (or mesh cuts) to filter cell clumps. Use DPBS for murine cells and cell culture media for human cells.
3. Wash cells at $300 \times g$ for 10 min, remove supernatant, tap tube to loosen the pellet, and proceed with red blood cell lysis. Murine cells sustain well hypotonic shock. For that, tap tube to loosen cell pellet, add 18 mL of sterile DI water, mix for 5–10 s, and add 2 mL of $10\times$ Ca^{2+} and Mg^{2+} -free DPBS. Mix, add sterile DPBS to 50 mL, and wash for 10 min at $300 \times g$. For human tissue cells, use homemade or commercial red blood cell lysis buffer according to the manufacturer's instruction.
4. Wash cells at $300 \times g$ for 10 min, remove supernatant, resuspend cells in cell isolation buffer, and filter them if needed (using cell strainer or mesh cuts),

and/or dissociate clumps by intensive pipetting. Calculate cell numbers and evaluate their viability using Trypan blue staining.

3.3 Human and Murine Treg, Teffs and APC Isolation

Avoid using samples if more than 10–15 % of dead cells are observed prior to Treg isolation. Such levels require troubleshooting to improve cell isolation techniques and may seriously compromise the purity of isolated cells, especially Tregs. You may apply the Dead cell removal kit (Miltenyi) or dead cell isolation method by Ficoll using corresponding standard protocols (not detailed here), but in most cases it leads to insufficient cell numbers for Treg isolation.

There are three options of experimental setup: first one is to isolate the CD4+CD25+ subset as Tregs, CD4+CD25– as Teffs and CD4– cells as APC. This modification may be performed for both human and murine cells, and requires just a corresponding CD4+CD25+ Regulatory T cell isolation kit (Miltenyi) for human or mouse cells. Follow the manufacturer's instructions and wash out CD4– depleted cells to use them as APC. Then, obtain CD4+CD25– Teffs and CD4+CD25+ Tregs on the second step of isolation.

Second option is to use an additional kit with CD3 MicroBeads (Miltenyi) for human cells, or mouse CD90.2 MicroBeads (Miltenyi) for murine cells. Follow the manufacturer's instructions. In that case APC will be depleted of CD3+CD8+ cells, which are active dividers. As a result, use of CD3-depleted APC instead of CD4-depleted APC will provide with better Treg suppression within the same Treg/Teff ratios. Serious drawbacks of this approach are the need for additional cells that cannot be used for Treg isolation, and the more expensive isolation procedure. However, for most murine experiments starting cell number is not an issue.

In both cases, when CD3- or CD4-depleted APC are used, they may be irradiated (100 Gy) prior to suppression assay. Irradiation of APC cells will help to stop their divisions and therefore will help to improve suppression by Tregs in the given Treg/Teffs ratios. Another way to obtain a better suppression is to use slightly less APC if they are CD4-depleted, and about 1.3–1.5 times more APC if they are CD3-depleted.

Third option is to use CD4+CD25+ Regulatory T cell isolation kit exclusively to obtain Tregs, and use a bulk of allogeneic or autologous splenocytes or lymph nodes (mouse) or PBMC (human) cells as responders and APC. There are different advantages of this strategy. First of all, it allows to standardize suppression assay by using an aliquoted standardized responders from the same healthy donor (*see* Subheadings 4.2 and 4.3 in Results). Second, the suppression effect of Tregs on CD4+ and CD8+ T cell divisions can be evaluated within the same assay. The drawback of this approach is to higher risk to receive a bad shape of CFSE peaks (*see* Subheading 4.5 in Results). Another drawback is the tendency of bulk cells to be more resistant to Treg suppression in comparison with CD4+CD25– Teffs and APC setup. To overcome this, increase Treg to responders ratio and adjust stimulation to the level of 0.6–0.9 times less from Teffs + APC setup levels (also *see* Subheading 4.3 in Results).

3.4 Labeling of Responder Cells

CD4+CD25- T effs or PBMC cells should be labeled with CFSE or its analogs according to the manufacturer's instructions. Avoid light exposure of cells. By the end of labeling, no changes in cell viability should be observed. After culture, labeled cells should demonstrate the same number and viability as non-labeled cells within the same experiment. If cells demonstrate decreased viability, then increase concentration of protein in labeling and/or washing buffers. You may also decrease concentration or time of labeling.

3.5 Suppression Assay

1. Calculate number of isolated Tregs, T effs, and APC (or Tregs and (PBMC). Numbers of T effs and PBMC should be evaluated after CFSE labeling. T effs and PBMC should be kept in dark after labeling and until plating. Dilute cells to 1×10^6 /mL concentration in cell culture media. If low number of cells used, prepare them in 0.5×10^6 /mL concentration to decrease pipetting errors (also see Subheading 4.3 in Results).
2. In advance, prepare CD3-coated magnetic beads to stimulate human cells by coupling of M-450 Tosylactivated beads (Invitrogen) with MACS GMP CD3 antibody (Miltenyi) according to the manufacturer's instructions in the bead package. Keep sodium azide-preserved sterile aliquots of the CD3-beads at 4 °C. Do not apply vortexing and vigorous pipetting for these beads. Such beads are stable for at least 12 months. In preliminary experiments, find the best stimulation for current experimental conditions (see Subheading 4.3 Results for details). Prior to cell culture, take the required volume of CD3 beads and wash them 2–3 times using magnet and cell culture medium. Prepare stock of anti-mouse CD3 functional grade antibodies (4 µg/mL) in cell culture medium.
3. Plate cells into 96-well U-bottom cell culture plate (if $35\text{--}80 \times 10^3$ Tregs used in 1/1 ratios) or into V-bottom plate (if $20\text{--}35 \times 10^3$ Tregs used in 1/1 ratios). Prepare serial 2× dilution of Tregs, starting with 2/1 or 1/1 Treg/Teff ratio and until 1/16 to 1/32 ratio. Do not use single Treg/Teff ratio for the experiment (see in Subheading 4.3 in Results). Then plate APC and CFSE-labeled CD4- T effs or CFSE- labeled PBMC, each well should have the same number of labeled cells as the number of Tregs in 1/1 ratio. It is convenient to mix human responder cells (CD4+ T effs or PBMC cells) with corresponding number of washed CD3-coated beads prior to plating. For murine cells, add corresponding volume of pre-diluted anti-mouse CD3 antibodies to have final concentration about 1 µg/ mL. Add cell culture media to have equal total volume in each well, 200 or 250 µL. Also plate cells for positive control (responder cells without Tregs) and negative control (responder cells without Tregs and without stimulation).
4. Incubate cells for 3–4 days (murine) or 4–6 days (human) at 37 °C.

3.6 Purity Control of Isolated Tregs

Immediately after plating of cells for suppression assay, stain left-over Tregs for LIVE/DEAD (Invitrogen), then stain for superficial markers: CD4, CD25, and CD127, and after

fixation and permeabilization step, stain for CTLA4 and FOXP3, according to the manufacturer's instruction for FOXP3 antibody. Alternatively, freeze the small aliquots of isolated Tregs, consisting of 2000–10,000 cells, to stain them for listed markers later. This is convenient for clinical trials, when bulk of patients' Tregs evaluation is preferable. Evaluate phenotype of isolated Tregs with flow cytometry. These data serve as Treg purity controls in interpretation of suppression assay results (see Subheading 4.1 in Results).

3.7 Cryopreservation of Human and Murine Cells

Use CryoStor[®] CS5 cell cryopreservation media (Sigma) according to manufacturer's instructions for freezing, storage and thawing [9]. For healthy donor PBMC, the viability just after thawing should be about 98–99 %. Cells should also preserve the same phenotype and same division rate as they had before freezing. As an internal quality control, we use healthy donor PBMC cells in cell culture experiments only if they demonstrated less than 5 % of dead cells by Trypan blue evaluation after CFSE-labeling.

However, in clinical trials, some patients' cells may demonstrate notable levels of apoptosis (10–30 % after thawing), probably due to hyperactivation in vivo or due to the effects of immunosuppressive therapy in transplant recipients.

For Treg cryopreservation using very limited cell numbers, you may exclude all optional washing steps. Thus, 1 mL of CryoStor media, pre-cooled on ice, can be used directly to wash out all leftover Tregs (it is about 10–50 μ l in cell culture media) from their 15 mL tube, and to transfer washouts into cryotube. After thawing, Tregs may be stained in the same 15 mL tubes, to exclude cell losses by transfer. If a washing step could not be excluded, the supernatant should be removed by pipetting instead of pouring. With such modifications, we usually receive at least 500–1000 events in live cell gates for Treg evaluation from any leftover clinical Tregs (see Subheading 4.1 in Results).

3.8 Evaluation of Suppression Assay Results

Stain cells with CD4 and (optional) CD8 antibodies, fix them in 1 % formaldehyde if required, and proceed with flow cytometry. Set up FS-SS gate on live cells. CFSE-negative cells are Tregs and APC, and CFSE-labeled cells are responders. Gate on CFSE+CD4+ subset to evaluate CD4+ Tregs divisions, and on CFSE+ CD8+ (or CFSE+CD4-) subset to evaluate CD8+ Tregs divisions. If needed, use additional LIVE/DEAD staining prior to fixation. See Subheading 4.5 in Results for details.

3.9 Statistics and AUC Calculation

In Results, data are shown as min-max and mean $\pm$ SEM. For continuous variables with normal distribution, statistical significance was assessed by Student's *t*-test to compare two groups, ANOVA with Newman-Keuls multiple comparison post-test to compare three groups, and Pearson correlation assay to test correlations. For data not normally distributed, two groups of continuous variables were compared with the Mann Whitney *U* test, and three groups were compared with the Kruskal-Wallis test with Dunn's multiple comparison post-test. A two-tailed *p* value of <0.05 was considered statistically significant.

For AUC calculations (*see* details in Subheading 4.6, in Results), we used GraphPad Prism v.5.00. After calculation of standardized or normalized suppression (described in the main text), we chose “XY” table format, and placed numbers of Teffs per Treg as values on *X* axis and corresponding percent of standardized suppression as *Y* values column(s) (*see* corresponding examples in Table 3 and Figs. 8 and 9). For the subsequent calculations, we excluded all zero values of suppression in “no Tregs” ratios, and decreased variability of data by replacing all minor suppression data that were 3 % and less, to zero values. Therefore, nonsignificant and biologically irrelevant cellular variability such as 86 % vs. 89 % of divisions giving 1–2 % of standardized suppression had no effect on resulting data. Then we used an option “XY analyses, area under curve” with standard settings to obtain AUCs numbers, listed as “total peak area.”

3.10 Ethics Statement

Pediatric liver and kidney allograft clinical studies were approved by the respective Institutional Review Board of each Center: CHOP-08–006311_CR1, CCHMC-2009–0018, and HSC- 1000014523. Adult liver allograft studies were approved by the Institutional Review Board of the Hospital of the University of Pennsylvania (#810878). Animal studies (C57BL/ 6 and BALB/c mice, The Jackson Laboratory, Bar Harbor, ME) were approved by the Institutional Animal Care and Use Committee of The Children’s Hospital of Philadelphia (#2010–6–561).

3.11 Processing of Human Cells from Healthy Donors and Transplant Recipients

PBMC-enriched apheresis product was obtained by leukapheresis of healthy volunteer donors, via the University of Pennsylvania Human Immunology Core. Specimens were collected according to the protocol approved by the Hospital of the University of Pennsylvania Institutional Review Board, and informed consent was obtained from each donor. For pediatric liver and kidney allograft studies, PBMC were isolated from 32 mL of blood and shipped overnight at 4 °C to the Children’s Hospital of Philadelphia (CHOP). For standardization, CHOP samples were also kept at 4 °C overnight. For adult liver allograft studies, PBMC were isolated from 45 to 50 mL of blood within a few hours of venipuncture, and kept overnight at 4 °C in cell culture media plus EDTA.

For all human Treg studies in this chapter, Tregs were isolated from PBMC using CD4+CD25+ regulatory Treg kits (Miltenyi Biotec). CD4+CD25- cells (Teffs) and CD4-depleted cells (APC), isolated with the same kit, were used in experiments with autologous cells. For some experiments, Teffs and APC were further cryopreserved to study combinations of autologous vs. allogeneic responders, or pre-transplant vs. post-transplant responders. In addition, for some experiments with healthy donor Tregs and responders, we used CD3-depleted and irradiated APC, isolated with CD3 MicroBeads (Miltenyi Biotec) according to the manufacturer’s instructions. For all clinical studies, we did not irradiate APC.

CD4+CD25- Teffs, or aliquots of cryopreserved healthy donor PBMC, were CFSE-labeled, and stimulated with CD3-coated beads (OKT3 clone from Miltenyi, Dynabeads® M-450 tosylactivated beads from Invitrogen) using a 3.5:1 bead:cell ratio in most cases. With some

responder cells, the optimal stimulation was adjusted in additional experiments, as described in Results, Subheading 3.3. Tregs and responder cells were cultured for 3–5 days (for clinical studies, we used 4 days) in cell media, consisting of RPMI-1640 plus 10 % heat-inactivated FBS, penicillin and streptomycin, and 2-mercapto-ethanol (100 μ M). CD4+ and CD8+ cell divisions were determined by CFSE dilution. When noted, we co-stained Tregs and responder cells with additional markers of interest after suppression assay or labeled Tregs with CellTrace (Invitrogen) prior to suppression assay. We used 96-well U-bottom plates, and for experiments with low cell numbers (35,000 and less of human Tregs in 1/1 ratio) we used 96-well V-bottom plates.

4 Results

4.1 Treg Isolation and Purity Control

4.1.1 Need to Evaluate FOXP3 in Isolated CD4+CD25+ as Treg Purity Control—

For many laboratories, the most common method of Treg isolation involves use of magnetic bead kits with CD4+ and CD25+ (high) markers. In normal murine samples, this method provides a stable and relatively pure (71–81 %, mean 77.1 ± 1.3) population of FOXP3+ cells, and CD4+CD25+ Treg isolation from healthy human donor PBMC can also be performed with satisfactory results (49–79 %, mean 64.7 ± 2.5), as detailed [10]. However, in murine models of autoimmune or inflammatory diseases, a significant percent of CD4+CD25+ cells are FOXP3- activated T effs. In murine models, use of fluorescent tags, such as YFP/GFP-FOXP3 mice [3], can address this problem, but isolation of Tregs from clinical samples is restricted by use of nonspecific surface markers. Despite this, most human Treg studies do not provide data of FOXP3 expression in isolated human CD4+CD25+ cells, and are thus of limited value. Figure 1a shows two human Treg samples isolated sequentially, as indicated, from the same liver allograft recipient. In both cases, CD4+ and CD25+ purity after isolation was reasonably high and comparable (78–82 %), but the first sample had no suppressive function at all due to a very low percentage of FOXP3+ Tregs within isolated CD4+CD25+ cells (see below). Of note, this patient was diagnosed with acute allograft rejection 7 days after the blood sample collection. This example illustrates the need for human clinical Treg samples to be evaluated for not only CD4+CD25+, but, at least, for FOXP3 purity after isolation, to allow interpretation of suppression assay findings.

The Treg number is usually claimed to be one of the most limiting factors for such additional tests, but in our studies, we found that it is possible to freeze, thaw and evaluate Treg aliquots of just 2000–10,000 cells, and to perform good quality staining for CD4, CD25, and FOXP3 as well as any other Treg-associated markers of interest (see Subheading 3.3, and Fig. 1a and [10] for examples). This number of cells typically requires just a washout from tubes after the majority of Tregs were used for suppression assays.

4.1.2 Role of CTLA4 and an Absence of CD127 as Treg-Associated Markers—

To improve Treg/FOXP3+ purity in human samples after isolation, some additional and potentially useful markers have been suggested. In our experience, CTLA4+ and CD127- had the most significant correlation with suppressive function of corresponding isolated

Tregs (Fig. 1b and [10]), but use of these markers in clinical studies is not reasonable, due to extremely low Treg yield and inconsistent results for corresponding FOXP3+ expression (described in detail [10]). As a compromise, we included CD127 and CTLA4 expression in our current panel evaluating purity of Tregs after isolation. In a study liver transplant recipients, CD127 expression in isolated Tregs inversely correlated with all other Treg-associated markers, including MFI of FOXP3 (Fig. 1b). Moreover, CD127 also inversely correlated with stability of FOXP3 expression, evaluated as FOXP3+ in Tregs after 4 days of suppression assay, with suppressive function for CD4+ and CD8+ responders and with FOXP3 demethylation in the Treg-specific demethylated region (TSDR) (Fig. 1b).

4.2 Choice of Responder Cells for Suppression Assay

4.2.1 Problems with Use of Autologous Responder Cells in Clinical Samples—

Use of autologous responder cells is often thought to be more physiologically appropriate, and avoids potential alloreactivity. However, autologous responders in Treg suppression assays have grave shortcomings that may lead to unsatisfactory results. First, a disadvantage of autologous responders is their dependence on pre-existing in vivo conditions. Multiple inflammatory and metabolic factors, medications and unidentified factors may affect the ability of CD4+ and CD8+ T cells to divide, as well as their resistance to Treg suppression, and also the costimulatory function of autologous APC. For example, we have noticed in our studies of liver transplant recipients that CD4+ Teff cell division was inversely correlated with total bilirubin levels in corresponding blood samples (Fig. 2a). We also observed a tendency to impaired cellular division in autologous CD4+ Teffs stimulated with CD3 and autologous CD4-depleted irradiated APC in liver transplant patients with hepatitis C, in comparison with the non-hepatitis group (data not shown). As a result of multiple complex factors, CD4+ Teff cellular division from liver transplant recipients did not reach levels obtained with healthy donor controls, before or after transplantation (Fig. 2b). Another problem may arise from technical factors, such as loss of co-stimulatory activity in autologous APC, isolated from overnight-shipped whole blood in multi-center clinical studies (Fig. 2c). The in vivo effects of immunosuppressive therapy in freshly isolated early post-transplant samples can become so prominent that autologous responder cells may become unable to divide with or without the additional impact of Tregs (Fig. 2d).

Importantly, differences in responder cell division rates are problematic in Treg suppression assays, and not off-set by standardization formulas that subtract responder cells divisions in the presence of Tregs from responder cell division in the absence of Tregs, divided by divisions without Treg. By this calculation, the suppressive function of Tregs becomes substantially overestimated in assays with low baseline responder cell division, and vice versa (Fig. 2e and reviewed in ref. 11). Therefore, use of autologous responder cells in clinical Treg studies can be misleading. Any condition that affects the ability of the responder cells to divide can skew the measured suppressive Treg function and lead to overestimation of Treg suppressive function (Fig. 2f).

4.2.2 Use of Healthy Donor Responders—In contrast to autologous Teffs, healthy donor responder cells are convenient for standardization, when multiple small aliquots ($1-3 \times 10^6$ cells per tube, 100–200 vials for study) of PBMC from the same donor may be

preserved in liquid nitrogen. In preliminary experiments, we tested PBMC from different donors and the same Tregs, and found that any effect of HLA mismatch for human suppression assays with polyclonal CD3 stimulation was negligible. In other words, “strong” Tregs remained very suppressive with any healthy donor’s responder cells, and vice versa. Therefore, to assess patient Treg suppressive function objectively, the suppression assay should be standardized as efficiently as possible, excluding potential confounders from autologous Teffs, leaving patient Tregs to be the only remaining variable in the experimental conditions.

We have noted the practical value of using healthy donor PBMC responder cells in our pediatric allograft cross-sectional study, given the impaired Treg function in patients with higher calcineurin inhibitor (CNI) levels in comparison with low-CNI levels, and correlations of Treg suppressive function with their CTLA4+ and CD127-phenotypes [10]. In addition, ongoing studies of adult liver transplant recipients indicated additional benefits of using standardized, healthy donor PBMC responder cells. Thus, these patients’ Tregs showed a marginal suppression with autologous responder cells, but an absence of any suppression, or even some stimulation, with healthy donor PBMC responder cells at 7 days prior to an episode of acute rejection (Fig. 2g). The phenotype of these cells after isolation is shown in Fig. 1a. We found that 10 % of remnant FOXP3+ cells were still able to suppress proliferation of in vivo immunosuppressed autologous responders, while normal responder cells provided far more plausible and clinically relevant results, namely decreased Treg suppressive function 1 week ahead of an acute rejection episode.

4.2.3 Use of Combinations of Autologous Cells to Obtain Additional Data—

Using combinations of a patient’s Treg with cryopreserved autologous responder cells can be useful to study the effects of immunosuppression protocols, including responder cell resistance to Treg suppression. Thus, 3 months after the episode of acute rejection, Tregs from the patient depicted at Figs. 1a and 2f were 54 % FOXP3+ after isolation, and restored their suppressive phenotype with healthy donor responder cells (Fig. 3a, top row). A hyper-activated phenotype of cryopreserved pre-rejection CD4+ Teffs from this patient led to substantial apoptotic cell death and impaired cell division using autologous, post-rejection APC (seemingly, due to shortage of co-stimulation for alloreactive clones). However, use of pre-rejection APC in combination with pre-rejection CD4+ Teffs not only improved Teff viability and partially restored their division rate, but also showed enhanced resistance to Treg suppression post-rejection (Fig. 3a, middle and bottom rows, respectively).

Cryopreservation of non-hyper-activated autologous cells is usually free from additional pro-apoptotic effects. These cells survive freezing and thawing well and exhibit neither signs of impaired viability nor defects in proliferation. Thus, comparison of suppression curves of post-transplant CD4+ Teffs with their pre-transplant counterparts (Fig. 3b, top and second rows) clearly shows that post-transplant CNI- and steroid-treated Teffs have diminished capacity for cell division. At the same time, pre-transplant Teff cells do not demonstrate enhanced resistance to post-transplant autologous Treg suppression (i.e., 1/1 and 1/2 ratios are the same despite enhanced proliferation at low Tregs ratios). If post-transplant APC is replaced by pre-transplant APC, free of in vivo immunosuppression effects (Fig. 3b, top vs. third rows), suppression curves show evident increases in cellular resistance to Treg

suppression with the same divisional capabilities of post-transplant CD4⁺ Teffs. This effect can be further studied, e.g., using co-stimulation blocking antibodies, analysis of pre- vs. post-transplant APC phenotypes and their cellular composition, and by investigation of soluble factors released into the cell media. Finally, comparison of suppression curves with post-transplant APC and Teffs vs. pre-transplant APC and Teffs verified these observations, namely that pre-transplant responders had enhanced Teff division and increased resistance to post-transplant autologous Treg suppression. To study these mechanisms in detail, healthy donor Tregs can be used in parallel with patient Tregs and the same combinations of patient responder cells. At the same time, use of healthy donor responder cells can serve as an internal control, providing standardized conditions for evaluation of a patient's "polyclonal" Treg suppressive function.

4.3 Standardization of Conditions to Receive Comparable Results of Suppression Assays

4.3.1 Choice of Healthy Donor and Detection of Optimal Stimulation—In contrast to the near-uniform proliferative potential of murine responder cells that, under standard conditions, are only affected by technical factors (cell isolation, cell media, CD3 antibody vendor lot), human PBMC responders do not provide such consistency. In our experience, studies involving human Treg suppression assays require serial preliminary tests determining PBMC responsiveness to stimulation, in order to detect the optimum range of stimulation for each donor. The most ideal stimulatory condition for a Treg suppression assay is located slightly below the level where responder PBMC reach a plateau of maximal division. Thus, if a given donor's PBMC divisions are 70 % at 2/1 CD3-bead/cell ratio, 80 % at 2.5/1 and 85 % at 3/1 and above, then 2.5/1 may be the optimal stimulation for a Treg suppression assay.

A second important factor for donor selection is the high consistency of his/her PBMC divisions, evaluated in different cryopreserved aliquots obtained from the same blood sample. Moreover, cryopreserved cells should not demonstrate any significant differences from the corresponding freshly isolated cells, which ensure appropriate cryopreservation-thawing techniques and therefore limit inter-assay variability in experimental conditions. In our current cryopreservation method (Materials and Methods), the majority of healthy donors satisfy these criteria. Comparisons of PBMC from 2 to 5 different donors, with a range of stimulatory conditions within the same experiment, will help to detect persons whose cells are mostly distinct from other donors in their stimulation/ co-stimulation requirements. Usually, these donors are not an appropriate choice for use as standardized healthy donor responders as they often demonstrate significant variability after cryopreservation, impaired cell division, or enhanced resistance to Treg suppression. When the best donor and range of optimal stimulatory conditions are established, 2–3 suppression assays with autologous and allogeneic Tregs will help to adjust stimulatory requirements to ensure high division rate with low Treg ratio, and with suppression of division with high Treg ratios. In the following clinical study, division of cryopreserved aliquots of responder cells can be used to maintain standardized conditions for subsequent suppression assays. This is very useful when new aliquots of anti-CD3- coated beads need to be re-calibrated (Fig. 4a), or cell culture media or other reagents need to be replaced, etc.

4.3.2 Cryopreserved vs. Freshly Isolated Responder Cells—Taking into account the potential problems related to cryopreservation, the idea of using the same person as a source of freshly prepared standard PBMC responders for small trials (regarding that just 3 mL of blood is usually sufficient for this purpose) may be an attractive alternative. However, in our experience, healthy donor cells serially collected at different times may not yield comparable results (Fig. 4b). Thus, well-cryopreserved aliquots of the same PBMC specimen remain the better alternative for standardized investigation of Treg function.

4.3.3 Sensitivity of Suppression Assay Depends of Responders—The optimal power to detect differences in Treg suppressive function may be obtained from responders that showed active divisions without Tregs, and are easily suppressed at high Treg ratios. In Fig. 4c, Tregs from two healthy donors were evaluated in combination with sets of two responder cells. With #339 responders, #341 Tregs demonstrate substantially stronger suppressive function, while #345 responders, due to a “narrow” interval within 1/1–0 Treg/Teffs ratios, cannot serve as a sensitive tool for Treg evaluation. Importantly, as mentioned above, the mutual relation of “stronger” vs. “weaker” Tregs (i.e., #341 and #339 Tregs, respectively, here) did not change when combinations of autologous or allogeneic, resistant or easily suppressed cells were used; therefore, any appropriate healthy donors cells can be used for this analysis, with results differing only in sensitivity.

4.3.4 Use of Cryopreserved Human Tregs—For clinical trials, when arrival of new samples for functional Treg tests is often unpredictable, and can occur after hours or interrupt ongoing experiments, use of cryopreserved PBMC for Treg isolation (or use of isolated cryopreserved Tregs) with later functional evaluation at a convenient time is a highly desirable option. Unfortunately, we have found that while murine Tregs sustain cryopreservation well and do not demonstrate substantial differences in their suppressive function in vitro and in vivo (in this paper [12], part of in vitro tests and all in vivo Tregs tests we performed using cryopreserved murine Tregs), most of freshly isolated human Tregs demonstrate impaired suppressive function after cryopreservation (Fig. 4d). As we were unable to stabilize the suppressive function of cryopreserved human Tregs, even those derived from healthy donors, we aimed to avoid introducing such variability into our clinical trials. However, in our experience, in vitro -expanded human Tregs sustain cryopreservation well, and their suppressive function, as well response to epigenetic compounds, were comparable between fresh and cryopreserved counterparts [13]. Indeterminate changes in functional activity of freshly isolated vs. cryopreserved human Tregs does not preclude their use in experiments where the same cells are using as a control and testing subsets, as for studying of compounds affecting Tregs, providing that fresh Treg cells demonstrate similar results in verification experiments.

4.3.5 Use of Decreased Cell Numbers in Suppression Assay—Importantly, the number of available Tregs is one of the most critical limiting factors not only in clinical trials, but also in some murine disease models. Therefore, we tested suppressive function using different starting Treg numbers, while Treg/Teffs ratios were preserved. In our conditions, murine cells stimulated with irradiated CD3-depleted splenocytes and soluble anti-CD3 antibodies, had markedly diminished proliferation at concentration of APC 30,000

cells/well or less, likely by progressive deficiency of co-stimulation. If the concentration of murine APC was maintained at 50,000 per well, Treg suppression assays showed generally comparable results in conditions from 50,000 to 20,000 cells/ well at 1/1 ratios (Table 1). These results also suggest that the standard conditions of a murine Treg suppression assay, soluble factors produced by murine Tregs may operate locally in paracrine manner. However, they are unlikely to play an important role in modification of culture conditions affecting distant responder cells within the same well, since the shapes of Treg suppression curves did not differ when 50,000 or 20,000 Tregs were used in the same 200 μ l total volumes.

Results of similar cellular titration with human Tregs do not provide such reproducible results due to high variability, but we have not observed significant differences in suppression function at 50,000 to 35,000 cells/well. At smaller numbers, 15,000–20,000 cells/well at 1/1 ratio in V-bottom plates, human Tregs tend to show an enhanced suppression in comparison with higher cellularity controls, but pipetting inaccuracies in serial dilutions, and therefore variability of results within duplicates, increase. As a practical approach, we routinely use 30,000–35,000 human Tregs at a 1/1 ratio, which allows to obtain more data from clinical samples with restricted blood volume or from post-transplant immunosuppression lymphopenic samples, but still provides reasonable accuracy in cell counting and pipetting.

4.3.6 Use of a Single Treg-Responder Cell Ratio if Treg Cell Numbers Are Limiting—Shortage of available Tregs in clinical studies often lead to attempts of researchers to save cells using just one given ratio in suppression assay for all samples. In our opinion, this approach has significant shortcomings. First, equal Treg function at one given ratio does not mean true equal function. Thus, in high Treg/Teffs ratios, when suppression is maximal and responders are not resistant, “weaker” Tregs can reach the same levels of feasible suppression as the “stronger” Tregs, and apparent differences in that case can be observed only when number of Teffs per one Treg increases (examples with raw data, Supplemental materials of [12]). Conversely, at lowest Treg/Teff ratios, suppression curves from Tregs with different suppression capabilities may merge together due to already equally diminished effects of suppression (Fig. 4, C 1/16 ratios at two top rows). Therefore, use of serial Tregs/Teffs dilutions increase the resolution of suppression assays, by increasing data for comparison and by leveling results of small pipetting errors with low cell numbers. Integrating curves of suppressive function over all measured ratios may additionally increase statistical power (see below).

4.4 Common Problems with Drug Testing

4.4.1 Compound Has no Specificity for Tregs—Since therapeutic manipulation of Tregs is an important area of interest, the effects of numerous compounds on Treg function have been tested. Unfortunately, the design of some studies precludes unambiguous interpretation of the results. One of the most frequent problems is the absence of any experiments showing the specificity of a given compound on Tregs vs. other cells, such as Teffs, especially for a compound that compromises Treg function. Figure 5a shows the results of Treg suppression assay with a chemically active compound isolated from

Australian pigweed [14] and suggested to have a detrimental effect on Treg functionality. Murine Tregs, preincubated with as little as 400 nM of this compound, demonstrated impaired suppressive function in comparison with Tregs pre-incubated with inactive control. However, such results cannot be interpreted as a Treg-related or Treg-specific effect, as murine Teffs, pretreated with the same compound, also showed impaired division (Fig. 5b). Thus, despite the absence of evident cell toxicity at the tested concentrations of active compound, a given compound can impair T cell division rate and the functional ability of T cells without compromising their viability, and therefore does not specify a Treg-mediated effect, at least during these preliminary in vitro experiments.

4.4.2 Compound Directly Affects Responder Cells—Another common problem in study design using compounds suggested to enhance Treg function is the use of reagents that directly suppress T cell divisions and/or disrupt APC co-stimulatory function in the suppression assay, when all three cellular participants (Tregs, Teffs, and APC) are treated together. In that case, effects on Tregs (stimulation of function and therefore decrease of responder cell divisions) cannot be distinguished from effects on responders (direct inhibition of responder divisions). Figure 5c shows results of in vitro human suppression assay with increasing concentrations of the clinically approved HDACi, SAHA. A gradually decreased rate of Teff divisions corresponded with increased direct effect of SAHA on responder cells (clearly seen at zero-Treg ratios, Fig. 5c). Therefore, in this design, the data cannot be attributed to effects of SAHA on Treg function. For such experiments, it is reasonable to perform a series of preliminary tests to establish the concentration of compound that has no obvious effect on responder cell proliferation. For murine cells, these preliminary tests are usually not problematic since they usually yield a reasonably narrow range of optimal concentrations that maintain as long as animals, operator and assay conditions are unchanged. However, with human cells, we found very high individual variability in such concentrations (Table 2). To overcome this problem, in human Treg suppression assays we test each compound at 2–4 different concentrations, and choose the one that shows no effects on responders in the absence of Tregs. Even this design, carefully controlled for cell viability (toxic effects) and with elimination of results that are not related to Tregs (if direct effects on responders' division rates were observed), it is not ensured that effects are indeed Treg intrinsic. In that case, the validation experiments with Tregs, pretreated separately [13], and/or confirmatory in vivo experiments [12] with similar results, should be performed.

4.5 Readout of Suppression Assay

4.5.1 Thymidine Incorporation vs. CFSE Dilution—For decades, thymidine incorporation has been considered as a “gold standard” for MLR assays, and, subsequently, as a readout for Treg suppression assays. Multiple data showed an absence of Treg divisions upon anti-CD3 stimulation (or even with dual CD3/CD28 stimulation with soluble antibodies, plate-bound antibodies or with CD3/28 beads). These data led to assertions that all divisional activity, evaluated with thymidine incorporation, originated from Teff cells. However, this assumption of Tregs non-division is true only in the absence of Teffs in the suppression assay. In mixed cell populations, such as PBMC, lymph node cells or splenocytes (Fig. 6a), both murine and human Tregs divide well, and their division rate is

usually even higher than in CD4⁺ Teffs, and sometimes approaches the division rate of CD8⁺ responders. The same is true for Treg suppression assays: use of two different fluorochromes allows for the evaluation of the division of responder and Treg cells in murine or human assays [15]. Similarly to murine cells, Ki-67 staining is also highly positive in human Tregs after suppression assays, especially for cells with stable FOXP3 expression that retain the Treg phenotype during the 4–5 days of assay (Fig. 6b). Therefore, when thymidine incorporation is employed in Treg suppression assays, data reflect division of both Tregs and Teffs, with additional confounding factors such as different Treg/Teffs ratios and level of suppression toward Teffs. These factors cannot be separated by this technique. Another important concern is that thymidine incorporation provides data of cellular division evaluated just within the last day of assay, while maximal suppression may occur within the first hours of Treg/Teff contact [16]. In contrast to thymidine-based assays, dilution of CFSE (or similar reagents) allows evaluation of cell division by responders and also, if needed, Tregs. An investigator can also perform co-staining with CD4 or CD8 antibodies, or study additional responder subsets such as naïve/memory populations, subsets of cells with upregulated activation markers, and Tregs after suppression assay for any markers of interest.

4.5.2 Methods to Enhance Quality of CFSE Peaks—Various concerns can also arise using CFSE-based methodology. While forward-scatter/side-scatter (FS-SS) gating after a suppression assay is usually sufficient to discern dead vs. live cells, less satisfactory results may be obtained when biosafety regulations (as for clinical samples) or intracellular co-staining require the use of fixed cells. In these cases, use of fixable live-dead markers prior to fixation (Material and Methods) gives significantly improved flow cytometry data. For unfixed cells, results of cell division, when FS-SS live gated cells or FS-SS + live/dead gating strategy are used, give similar data (Fig. 6c) although FS-SS gating tends to slightly flatten suppressive curves (providing more divisions at high Treg ratios and less divisions in low Treg ratios in comparison with live/ dead gated subsets).

Use of pulse width gating to include only single cells for evaluation additionally improves the quality of CFSE peaks, and can be useful when tissue cells (splenocytes, lymph node cells) are used, or when toxic effects are expected. For human PBMC in standard suppression assay conditions, very few cells are not singlets. In our experience, the quality of CFSE peaks, making them still easily distinguishable by the end of an experiment, is usually not an issue in murine Treg suppression assays. However, human PBMC responders from some healthy donors and a considerable percent of patient PBMC do not divide synchronously, and therefore do not provide clearly distinguishable peaks. In that case, use of isolated CD4⁺ or CD8⁺ responders with irradiated, CD3-depleted APC, instead of CFSE-labeled PBMC, can improve the shape of CFSE peaks.

Due to difficulties with resolution of peaks, some researchers prefer to use the mean of CFSE fluorescence instead of identification of nondividing subsets. However, this approach suffers from a high level of variability: multiple factors (number of cells for labeling, protein content in cell media during labeling, temperature and time of labeling, age of CFSE aliquot stock solution, light exposure for CFSE and for labeled cells, cell viability and their time in assay) affect the brightness of CFSE signal, making it difficult to perform reliable inter-

assays comparisons that are often critical in clinical studies. CFSE brightness may be so labile as to compromise results even in the same experiment, when multiple concentrations of testing compound are compared with controls that were run previously. Here we show comparison of CFSE mean fluorescence (Fig. 6d) vs. CFSE peaks (Fig. 6e) after 20 min of routine room light exposure. When CFSE mean of fluorescence was used for evaluation, nondividing and once divided cells with highest CFSE fluorescent means lost their brightness faster, leading to shrinkage of suppression curves (and false decreases in Treg suppressive function) within as little as 20 min of light exposure.

For hampered CFSE-peak shapes, an additional co-staining with Ki-67 helps to accurately establish location of nondividing CFSE peak either for murine or human responders (Fig. 7a). A great advantage of this method is there is no need to routinely fix cells and co-stain them with Ki-67, and can be a result-saving strategy when exploratory flow cytometry shows hardly distinguishable CFSE peaks.

4.5.3 Effects of CD4+CD25- Gating on Responders—Since ideally most Teffs are CD4+CD25- and Tregs are CD4+CD25+, some researchers use CD25 co-staining and gate for the CD25- subset in CFSE+ Teffs to evaluate cell division. We consider this approach unsuitable since murine and human Teff cells easily upregulate CD25 after just overnight stimulation. Furthermore, with human T cells, CD25- gating will falsely increase Treg suppressive function since CD25+ Teffs have a greater division rate compared to those that retain the CD25- phenotype (Fig. 7b). The same is true for mouse C57BL/6 cells (Fig. 7c, left), but, interestingly Th2- biased BALB/c mice demonstrate the inverse; in these mice, CD25+ cells showed impaired cellular division during suppression assays, compared to CD25- T cells (Fig. 7c, right). Taken together, the use CD25+ or CD25- gating for responder T cells may provide additional readouts, but should not be used as a method to discern Teffs from Tregs.

4.5.4 Alternative Ways to Evaluate of CFSE Peaks: Division Index Calculation of Progenitors—Use of division index instead of percent of dividing cells, as described [17], may be an excellent alternative providing higher sensitivity, but it is valid only in conditions where all CFSE peaks *and* are easily visible and distinguishable from each other, which is often not the case with clinical samples. However, our AUC-based method to calculate Treg suppressive function as one number for all Treg/Teffs ratios may be applied for division index data as well as for percent of divisions (see below).

Another readout method in Treg suppression assays came from studies with cell lines and is based on the calculation of progenitors, as reviewed [17]. This method works on the assumption that each cell produces two daughter cells after division, and therefore the final number of cells in the last division peak is the result of this geometric progression with a denominator of 2. This method is appropriate for cell lines, where normally only a very small percent of cells stop division, and few die during an experiment under normal conditions. However, in our experience, primary cells always demonstrate noticeable levels of apoptosis, and also can stop after 1, 2, or 3 rounds of division due to multiple factors, just one of which is suppression by Tregs. The shape of typical CFSE peaks with murine or

human primary T cells, with reduced numbers of dividing cells in the last division rounds, demonstrates this point.

4.6 How to Calculate AUC for Treg Suppression Assay

4.6.1 Two Modifications of X-Axis Labeling to Increase Sensitivity of Assay—

Area-under-curve (AUC) calculations are commonly used with glucose tolerance tests and in pharmacokinetic studies. Mathematically, it is a definite integral, but for practical purposes most statistical software use trapezoidal approximations for calculation. Any standard statistical package can be used to obtain results of Treg suppressive function as a standard calculation of AUC. For this method, we use an assumption that serial dilution of Tregs in Treg/Tefts ratios is the equivalent of a time function on the x -axis, where concentration of a given medication in patient's plasma gradually decreases. Therefore, Treg suppression at any given ratio from 1/1 to 1/16 (number of Tefts per Treg) or lower, if needed, is plotted on the y -axis against corresponding ratio. It should be noted that in this modification, AUC is sensitive to variability of results in low Treg/Tefts ratios and therefore good for Treg suppressive curves where the greatest differences occur at low ratios. Conversely, if responder cells are sensitive to suppression, and raw Treg curves demonstrate the highest differences in suppression in high Treg/Tefts ratios such as 1/1 and 1/2 (or even 2/1), it may be reasonable to replace “number of Tefts per 1 Treg” on x -axis to numbers equivalent to degrees of Treg dilutions. Therefore, in first scenario (AUC is sensitive to low ratios) the x -axis is labeled as 1, 2, 4, 8 and 16, while in second scenario the x -axis has numbers 0, 1, 2, 3, and 4. Both modifications will yield similar results in terms of discrimination of “weaker” vs. “stronger” Tregs, but the degree of these differences would differ according to the initial shape of the raw Treg curves.

4.6.2 AUCs for Drug Use vs AUCs in Clinical Treg Studies—

Two additional modifications of AUC calculations of suppressive function include experimental designs with internal controls serial assessments of Treg function. Examples of the latter are evaluations of Treg suppressive functions in cross-sectional clinical studies [10], or calculations of Treg suppressive function in follow-up clinical studies, where each patient provides Treg samples at different times. For such studies, Tregs assays should be highly standardized within their conditions (see above) to ensure that resulting data will be intercomparable, and patient's Tregs would be the single main factor that differs between experiments. An example of AUC calculation for this type of studies is shown in Fig. 8. Here, raw data of four Treg suppression assays, evaluating Tregs from the same liver allograft recipient at different time points are shown. For these experiments, autologous CD4+CD25-responders were used, and 7 day post-transplant (post-Tx) data with maximal levels of immunosuppression clearly show impaired CD4+ division. Interestingly, in this patient, CD4+ T cells recovered their proliferative activity by 3 months post- Tx (Fig. 8a). Then, raw suppression data were re-calculated as standardized suppression using the formula $S = ((\text{divisions without Tregs} - \text{divisions in current ratio}) / \text{divisions without Tregs}) \times 100$, where S is standardized suppression [18]. Due to significantly impaired division of autologous cells at 7 days post-Tx, standardized suppression is evidently compromised: the small variability in cell divisions occurred at 1/8 Treg/Tefts ratio turned out to produce a high suppression peak after re-calculation (Fig. 8b). This example illustrates the trial design

issues discussed above, where autologous cells, if they have impaired division, cannot provide reliable Treg data but rather tend to lead to false-positive suppression findings, and, conversely, resistant autologous Teffs tend to lead to false negative assessment of Treg suppressive function. Figure 8c uses the same data as for Fig. 8b, but actual areas under two suppressive curves are shaded for illustrative purposes, and two corresponding AUCs values are provided in the legend. Clearly pre-Tx Treg suppressive function was better, although autologous cell results have to be validated with standardized conditions, i.e., with healthy donor responder cells. Finally, Fig. 8d shows results for all four suppressive curves calculated as AUCs.

Examples of experimental design with internal controls are tests with epigenetically active compounds [13], or WT vs. different KO mice [19], or control vs. disease groups of mice, i.e., experiments where Treg function is compared in experimental vs. control conditions. The experimental conditions for each independent experiment in that design may be not comparable with each other. Thus, different healthy donors Tregs with their own autologous responders may be used each time, mouse groups can differ in age, gender, duration of experiment, etc., but within a given experiment, all cells (except for Tregs) are the same. In that case, standardization of raw Tregs suppression curves may be calculated firstly as min-max normalization (see Fig. 9a and Table 3, Steps 1 and 2), and then transformed into standardized suppression using $Y_t = 100 - Y$ formula for standardization, where Y_t is standardized transformed suppression (in %), and Y is normalized suppression (see Fig. 9a – c and Table 3, Steps 2 and 3 for detailed example). Resulting AUCs (Fig. 9d) may be additionally calculated as a fold ratio to control Tregs' AUC (Fig. 9e), which provides an option to group results of different experiments into the final groups with p value calculations for statistical differences [13].

In summary, we have used this described AUC calculation method in murine and human studies for over 6 years, and found it to be convenient, sensitive and reproducible in any given Treg suppression assay design, as well as a viable method to standardize between different investigators.

5 Discussion

Assessment of Treg suppressive function remains one of the most reliable methods for identification of Tregs ex vivo, and also facilitates the evaluation of new Treg-associated markers, including surface molecules, cytoplasmic or nuclear factors, and epigenetic modifications. Within the past several years, many detailed and thoughtful protocols on the essential elements of Treg suppression assays have been published [11, 17, 18, 20–26]. Therefore, we focused this work on features of Treg suppression assays, especially with regard to clinical samples. We have described the importance of assay standardization, and provided a new method for reliably measuring and calculating Treg suppressive function.

There is a great variability in conditions of Treg suppression assays [17], and multiple factors may affect the results [11]. Historically, most Treg assays employed thymidine incorporation, which has exquisite sensitivity to detect differences using low cell numbers, and does not require access to a flow cytometer. Unfortunately, this method involves the

false assumption that Treg divisions do not compromise the final results due to their “anergic” status in vitro. In the current manuscript, and previously [15], we showed that murine and human Tregs indeed divide vigorously during Treg suppression assays, and the level of IL-2 produced by Teffs, even at a 1:1 Treg:Teffs ratio, is sufficient to overcome any “anergic” Treg state in vitro. Thus, only CFSE-based suppression assays [26] or analogs provide data on responder cell divisions that are free of contamination by Treg division. With regard to concerns of the sensitivity of CFSE-based assays, we showed that cell numbers can be reduced to 20,000–25,000 of responders/Tregs in 1/1 ratios without any adverse effects, provided cell numbers are consistent between different experiments in the same study. Moreover, with relatively low cell numbers (40,000 and less), we never observed artifactual suppression due to the competition of highly activated CD25+ non-regulatory T cells, isolated as Tregs, with responder cells for growth factors or stimulation, as discussed [17]. Conversely, in our studies, highly activated human non-regulatory T cells stimulated healthy donor responder cells to divide, or had no effect. Use of flow-based assays for evaluation of division of responder cells can provide researchers with opportunities to obtain significant additional data. Cell culture media may be collected to evaluate soluble factors (IL-10, IL-2, IFN- γ , IL-17), cells may be fixed and co-stained with antibodies against cytokines and for transcriptional factors, and they may also be studied for expression of maturation and cell lineage markers. For example, staining cells at the end of the Treg suppression assay for Helios allowed us to study the effect of the presence or absence of Helios on Treg and Teff cell function, proliferation, and Teff susceptibility to Treg suppression [15].

As Tregs employ diverse mechanisms for their suppression, which may be regulated by cytokine milieu and other uncharacterized environmental factors [27], and depend on the type of responder cells and ongoing immune processes [28], Treg suppression assays can be modified accordingly, allowing a focus on defined conditions. Use of allergens for stimulation in order to study allergies [23], or use of donor-specific alloantigens to study Treg abilities to suppress anti-donor immune response in transplant studies [29, 30] are examples of such modifications. However, the results of these modifications should be interpreted with caution, as they may not reflect true in vivo situations. Thus, direct alloantigen recognition plays a major role only in early stage of alloantigen responses, and is then replaced by indirect recognition [31, 32], and alloantigen repertoires may be significantly modified by post-ischemic transplant events and chronic graft deterioration. Outcomes may also vary based on use of a donor’s native APC, or donor-antigen loaded APC isolated post-Tx. By contrast, use of standard polyclonal stimulation, which affects all Teff and Treg subsets, in comparison with healthy controls or pre-Tx cells, may provide more reliable evidence of alterations in Treg functionality, and should be performed at least as a control in any types of specifically modified Treg suppression assays.

To conclude, we propose that the utilization of standardized and carefully controlled conditions of Treg suppression assays, along with AUC calculations, may significantly improve the reproducibility of published results and therefore help to further improve our knowledge of Tregs and their suppressive mechanisms.

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Fig. 1.

Treg phenotypes after isolation and assays of their suppressive function. **(a)** Tregs were isolated from the same liver allograft recipient 7 days prior (*left*) and 3 months after an acute rejection episode (*right*) and stained for live/dead, CD4, CD25, and FOXP3. Gated CD4+ cells demonstrate comparable CD25+ expression, but very low FOXP3 level in pre-rejection sample. **(b)** Tregs were isolated from 8 liver allograft recipients pre-transplant, and then at 7–14 days, 3 months, and 1 year post-Tx. Aliquots of isolated Tregs were cryopreserved and stained for live/dead, CD4, CD25, CTLA4, FOXP3, and CD127, while freshly isolated Tregs

were used for suppression assays with healthy donor's responder cells (PBMC). CD127 expression in isolated Tregs shows inverse correlations with many important Treg-associated markers and with suppressive function of these cells

Fig. 2.

Problems with use of autologous responder cells in Treg suppression assays. **(a)** Bilirubin levels in blood inversely correlated with Teff proliferation. CD4+CD25- Teffs were isolated from the same liver allograft recipients as in Fig. 1b (7–14 days and 3 months post-Tx), and stimulated with autologous CD4-depleted APC and anti-CD3 beads at 3.5/1 beads/Teff cell ratio for 4 days. Data of Teff proliferation was plotted against corresponding total bilirubin levels obtained at the same time point. **(b)** Allograft recipient CD4+ Teffs demonstrated impaired divisions in comparison with healthy donors cells. CD4+CD25- cells isolated from

the same patients as shown at Fig. 1b (pre-Tx or 7–14 days and 3 months post-Tx) were stimulated as in (a), and their rates of division were compared with healthy donor CD4+CD25- Tregs stimulated under the same conditions. ANOVA with Newman-Keuls multiple comparison test was used for comparison. (c) Whole blood sample from a patient listed for lung transplant was shipped overnight at room temperature, and CD4+CD25- cells were isolated and stimulated as in panels (a) and (b). Four days later, Tregs were alive, but showed a lack of proliferation (top). At day 4, CD3/28 beads were added at 1/1 bead/cell ratio, and 4 days later Tregs demonstrated restored proliferation. (d) Treg suppression assay was performed with Tregs from liver transplant allograft recipient, with autologous Tregs and APC, stimulated as in panel (a). Autologous cells, collected 7 days post-Tx, exhibited a lack of proliferation, making evaluation of Tregs suppressive function impossible. (e) Results of autologous suppression assays of healthy donors and liver transplant patient Tregs (pre-Tx) were grouped according to maximal rate of CD4+CD25- divisions. When Tregs had impaired proliferation, corresponding Treg function seemed to be higher. Data were analyzed by Mann-Whitney test. (f) Results of autologous suppression assays of healthy donors and liver transplant patient Tregs (pre-Tx and 3 months post-Tx) were compared. Healthy donor Tregs tended to demonstrate worse suppressive function than patient Tregs, although data did not reach significant *p* value (Kruskal-Wallis test). (g) Tregs depicted in Fig. 1a on left, demonstrated marginal suppression in assay with autologous (top), but not with healthy donor responders (bottom). For all data, * *p* < 0.05 and ** *p* < 0.001

Fig. 3.

Use of different combinations of autologous or allogeneic responders to evaluate Treg function (a) Tregs shown in Fig. 1a (*right*) demonstrated suppressive function with healthy donor responders (*top*). Combination of pre-rejection autologous Teffs (*middle*) or pre-rejection autologous Teffs and APC (*bottom*) showed that pre-rejection APC provided better co-stimulation for Teffs and some resistance to Treg suppression in comparison with 3 months post-Tx APC. (b) Combination of 3 months post-Tx Tregs either with autologous responders collected the same time (*top*) or pre-Tx. Pre-Tx Teffs demonstrate better

divisions (*second row*), pre-Tx APC confer increased resistance of Teffs to Treg suppression (*third row*), and combination of both pre-Tx responders (Teffs and APC) demonstrate sufficiently enhanced Teff cell proliferation, as well as increased resistance to Tregs (*bottom row*)

Fig. 4.

Factors affecting standardization of Treg suppression assays. **(a)** Tregs isolated at 3 months post-Tx from a liver allograft recipient demonstrated some suppression with healthy donor's responders when stimulated with current aliquot of anti-CD3 beads (*top*), but a new aliquot of anti-CD3 beads used in the same 3.5/1 ratio, clearly over-stimulated responder cells, which led to extinction of Treg suppressive function (*bottom*). **(b)** PBMC from the same healthy donor, collected with 1-year period, demonstrate differences in their divisions. Both PBMC samples were cryopreserved and used within the same experiment. **(c)** Selection of

the best responder cells for human Treg suppression assay. While use of both #339 and #345 responders demonstrate that #341 Tregs have better suppressive function than #339 Tregs, responder cells with larger range between 1/1 and 0 Tregs ratios (*top two rows*, #339 responder) provide better sensitivity to discriminate Treg function. **(d)**. Impaired function of human Treg function after cryopreservation. While cryopreserved human Tregs were viable and had no evident changes in their phenotype when evaluated by flow cytometry after thawing, they demonstrated impaired suppressive function

Fig. 5.

Use of Treg suppression assay to evaluate compounds suggested to affect Treg function. Murine Tregs (**a**) or Teffs (**b**) were preincubated with a chemically active compound isolated from Australian pigweed plant, or with control inactive compound, for 2 h, washed twice and tested in a Treg suppression assay. In preliminary experiments, 400 nM of compound did not show any evident toxic effects during 3 days of culture of murine splenocytes. While data show impaired Treg suppression (**a**), additional data (**b**) demonstrated impaired division of pretreated CD4⁺CD25⁻ Teffs, suggesting the effect of the compound is not specific for Tregs. Each experiment was performed twice with different concentrations of compounds and similar results. (**c**) Human Tregs, CD4⁺CD25⁻ Teffs and CD3-depleted irradiated APC were incubated with different concentrations of SAHA for 4 days. As the concentrations of

SAHA used directly inhibited Teff division, a finding of enhanced Treg function due to SAHA treatment cannot be distinguished from direct inhibitory effect of SAHA on responder cells, and therefore no reliable conclusions can be derived from such experiments

Fig. 6.

Different approaches to the analysis of Treg suppression assay data. **(a)** Human (*top*) and murine (*bottom*) Treg division in mixed cell populations consisting of PBMC (*top*) or splenocytes (*bottom*). Cells were stimulated with CD3, without addition of IL-2. Percent of dividing cells is shown. **(b)** Human Treg division in 4 day suppression assays, 1/1 ratio. Tregs with stable FOXP3+ phenotype upregulated Ki-67 better than Tregs that lost FOXP3. **(c)** Differences in cell division when live cells were gated according to FS-SS properties (*black histograms*), or when FS-SS gating was complemented with live/dead staining (*grey*

histograms). Differences in CFSE mean fluorescence (**d**) or in percent of dividing cells (**e**) of the same cells after 20 min of light exposure of murine cells in Treg suppression assay

Fig. 7.

Readout of CFSE- based suppression assay. (a) Co-staining with Ki-67 helps to locate the first CFSE peak of nondividing cells in human (*left*) or murine (*right*) suppression assays. (b) In human suppression assays, Teffs cells that upregulated CD25 are better dividers than CD25- cells. Healthy donor (*left*) or pediatric liver allograft patients (*middle* and *right*) Tregs were used in suppression assays with autologous CD4+CD25- Teffs (*left*) or with healthy donor PBMC (*middle* and *right*) as responders cells. 4 days later, cells were stained for CD4 and CD25, and CD25+ cells were plotted vs. CFSE in CD4+ gated cells. Tregs are located

on left two quadrants of each dot plot. (c) In murine Treg suppression assays using cells from C57BL/6 mice, CD4+CD25+ responder cells demonstrate better divisions than CD25- Tregs (*left*), while using cells from BALB/c mice, cells with upregulated CD25 proliferated less than CD4+CD25- Tregs (*right*). Each murine experiment was performed twice with the same results

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Fig. 8.

Calculation of standardized suppression and AUC for Treg assays in clinical Treg studies.

(a) Raw data showing CD4+ Teff proliferation in four autologous suppression assays, performed with samples from the same liver allograft recipient. Cells were isolated preoperative (Pre-op), and at 7 days, 3 months, and 1 year post-Tx. (b) Standardized suppression (S) was calculated using $S = ((\text{divisions without Tregs} - \text{divisions in current ratio}) / \text{divisions without Tregs}) \times 100$. (c) Two areas under the standardized suppression curves, as in (b), are shaded for illustrative purposes. Corresponding AUC values are shown in graph legend on right. (d) Suppressive function of four Treg samples for autologous CD4+ responders, calculated as AUCs

Fig. 9.

Calculation of standardized suppression and AUC for Treg assays with epigenetic compounds. Healthy donor CD4⁺CD25⁺ Tregs, CD4⁺CD25⁻ Teffs, and CD4-depleted irradiated APC were used in a suppression assay with different concentrations of valproic acid (HDACi), RG108 (DNMTi), and SAHA (HDACi). Then, one concentration of each compound, which demonstrated no direct inhibitory effect of given compound on Teff divisions, was chosen for following calculations. **(a)** Raw data show division of CD4⁺ Teffs at corresponding Treg/Teff ratios. Data are plotted as number of Teffs per (one) Treg vs.

corresponding division of CD4+ T effs. Table 3, Step 1 shows the same data as % of dividing CD4+ T effs. **(b)** Within this experiment, the lowest and the highest divisions (bolded in Table 3, Steps 1 and 2) were used to define 0 and 100 % for percentage normalization. A screenshot of the dialog box from GraphPad Prism with corresponding data is shown. **(c)** Data from Table 3, Step 3, are plotted. Normalized suppression data were transformed using formula: $Y_t = 100 - Y$, where Y_t is transformed normalized suppression, and Y is normalized division. Y values show normalized and transformed suppression. All values in ratios without Tregs were excluded from further AUC calculations. **(d)** Treg suppressive function, calculated in AUCs units, is shown. All compounds clearly enhance Treg suppressive function. **(e)** The same data as **(d)** were re-calculated with control Treg suppressive function as denominator. As a result, data shown fold increase of Treg suppressive function for each compound vs. control (which is 1). These data may be further grouped with results of similar suppression assays with other Tregs and responders, to compare means of enhanced Treg function and p value for differences

Table 1

Effects of different starting numbers of cells in murine Treg suppression Assays

Concentration, cells per well	Divisions of CD4 ⁺ Teffs at corresponding Treg/ Teff ratio, %				
	1/1	1/2	1/4	1/8	0
50,000	29	45.4	67.2	77.4	93.7
40,000	27.8	32.3	58.1	75.9	94.8
30,000	20.9	35.8	63.1	80	96.4
25,000	30.8	41.6	47.5	73.1	97.3
20,000	26.8	42.5	60	79.8	95.7

Treg suppression assays were performed using 20, 25, 30, 40, and 50×10^3 of Tregs/ Teffs per well in 1/1 ratios. Soluble anti-CD3 mAbs were added to final concentration of 1 $\mu\text{g}/\text{ml}$, and each well had 50×10^3 of irradiated CD3- depleted APC. The final volume of culture media in each well was equal to 200 μl , and the same U-bottom plate was used. Values that considerably differ from corresponding counterparts are bolded. This experiment was performed twice with similar results

Table 2

Results of preliminary tests to establish range of concentrations of epigenetic compounds that do not affect Teff division in the absence of Treg cells

Decitabine (DNMTi)		Valproic acid (HDACi)					
Donor and results: toxic effect, %		Donor and results: toxic effect, %					
Donor1	Donor 3	Donor 5	Donor 1	Donor 2	Donor 4	Donor 5	Donor 6
15.6 nM	<i>n/t</i>	0	15.6 μ M	<i>n/t</i>	<i>n/t</i>	0	<i>n/t</i>
31.25 nM	<i>n/t</i>	7.6	31.25 μ M	<i>n/t</i>	2.1	0	3.5
62.5 nM	<i>n/t</i>	5.8	62.5 μ M	<i>n/t</i>	7.6	<i>n/t</i>	3
0.125 μ M	<i>n/t</i>	9.34	0.125 mM	<i>n/t</i>	10.5	<i>n/t</i>	2.7
0.25 μ M	<i>n/t</i>	14.7	0.25 mM	<i>n/t</i>	25.4	5.6	7.8
0.5 μ M	<i>n/t</i>	17.3	0.5 mM	11.5	<i>n/t</i>	13	<i>n/t</i>
1 μ M	<i>n/t</i>	19.7	1.0 mM	46.5	<i>n/t</i>	<i>n/t</i>	<i>n/t</i>
3.42 μ M	8.4	<i>n/t</i>	<i>n/t</i>				
6.84 μ M	14.7	<i>n/t</i>	<i>n/t</i>				
13.69 μ M	10.4	<i>n/t</i>	<i>n/t</i>				
27.38 μ M	15.5	<i>n/t</i>	<i>n/t</i>				
54.75 μ M	17.4	<i>n/t</i>	<i>n/t</i>				

Healthy donor's CD4+CD25⁺ Teffs were CF SE labeled, incubated with irradiated autologous CD4-depleted APC and with listed concentrations of compounds. Differences between divisions rate in control (no compound) vs. tested concentrations are shown as "Toxic effect, %", "n/t"—not tested

Table 3

Calculation of enhanced Treg suppressive function in an experiment with epigenetic compounds

Step 1. Raw data: divisions of CD4+ cells

Number of Teffs per Treg	Divisions of CD4+, %			
	Control	Valproate	RG108	SAHA
1	46.30	31.80	25.0	37.30
2	46.60	35.50	46.0	38.30
4	70.10	51.20	55.8	44.80
8	77.30	63.30	62.1	67.90
16	76.30	72.80	74.2	71.30
0	88.70	89.20	87.5	87.60

Step 2. 0–100 % Normalized data

Number of Teffs per Treg	Normalized divisions of CD4+, %			
	Control	Valproate	RG108	SAHA
1	33.18	10.59	0.00	19.16
2	33.64	16.36	32.71	20.72
4	70.25	40.81	47.98	30.84
8	81.46	59.66	57.79	66.82
16	79.91	74.45	76.64	72.12
0	99.22	100.00	97.35	97.51

Step 3. Transformed data

Number of Teffs per Treg	Transformed normalized data, suppression, %			
	Control	Valproate	RG108	SAHA
1	66.82	89.41	100.00	80.84
2	66.36	83.64	67.29	79.28
4	29.75	59.19	52.02	69.16
8	18.54	40.34	42.21	33.18
16	20.09	25.55	23.36	27.88
0	<i>0.78</i>	<i>0.00</i>	<i>2.65</i>	<i>2.49</i>

Healthy donor CD4+CD25+ Tregs, CD4+CD25- Teffs, and CD4-depleted irradiated APC were used in a suppression assay with different concentrations of valproic acid (HDACi), RG108 (DNMTi), and SAHA (HDACi). Then, one concentration of each compound, which demonstrated no direct inhibitory effect of given compound on Teff divisions, was chosen for following calculations. In Step 1, raw data show division of CD4+ Teffs at corresponding Treg/Teff ratios. Within this experiment, the lowest and the highest divisions (bolded) were used to define 0 and 100 % for percentage normalization in the next step. For Step 2, the smallest CD4+ division value in current experiment defined as 0 % and biggest one as 100 % (both are bolded). Corresponding dialog box in Prism Graph Pad is shown at Fig. 9b. For Step 3, data transformed as $Y_t = 100 - Y$, where Y_t is transformed normalized suppression, and Y is normalized division. For further calculations, data of “0 Treg” ratios are excluded (shown in italic)