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Immunosuppression in breast cancer: a closer look at regulatory T cells

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Flow cytometry-based isolation of tumor-associated regulatory T cells and assessment of their suppressive potential

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ABSTRACT

Regulatory T cells (T_{regs}) play a major role in establishing an immunosuppressive tumor microenvironment. In order to fully uncover their role and molecular regulation in tumor-bearing hosts, it is critical to combine phenotypical characterization with functional analyses. A standard method to determine the suppressive potential of T_{regs} is with an *in vitro* suppression assay, in which the impact of freshly isolated T_{regs} on T cell proliferation is assessed. The assay requires the isolation of substantial numbers of T_{regs} from tissues and tumors, which can be challenging due to low yield or cell damage during sample preparation. In this chapter we discuss a flexible suppression assay which can be used to assess the suppressive potential of low numbers of murine T_{regs}, directly isolated from tumors. We describe methods for tissue preparation, flow cytometry based sorting of T_{regs} and optimal conditions to perform a suppression assay, to obtain reliable and reproducible results.

INTRODUCTION

There is a growing appreciation for the influential role of the tumor microenvironment on cancer biology[1]. This has led to the realization that the immune system is not only involved in tumor clearance, but can also support tumor growth and metastasis via different mechanisms, including the induction of intra-tumoral and systemic immunosuppression[2]. One of the key cell types that is involved in exerting immunosuppression in cancer patients is the $Foxp3^+$ $CD4^+$ regulatory T cell (T_{reg})[3].

In mice, regulatory T cells are characterized by high expression of the transcription factor Foxp3, which arms these cells with immunosuppressive properties[4,5]. Other proteins that are highly expressed by T_{regs} include CD25, CTLA-4 and GITR[6]. T_{regs} play a central role in many aspects of immune homeostasis, and are for example critical in resolving inflammation, dampening immunity towards food- and commensal microbial antigens and controlling adipose inflammation[7–9]. Importantly, T_{regs} are the gate keepers of peripheral tolerance and thus responsible for clearing auto-reactive T cells from the periphery[10]. To perform this role, T_{regs} have a wide arsenal of immunosuppressive abilities, including the release of cytokines, inhibition of T cell priming and direct effector cell killing[6]. In a cancer setting, many of these immunosuppressive mechanisms can be directly employed to prevent anti-tumor immunity and promote tumor growth[3]. However, the type of suppressive effector mechanism that is used in a certain setting depends on many factors, such as signals from the local environment, and the activation status of the T_{reg} or the target cell. Therefore, when investigating T_{regs}, it is important to combine a comprehensive phenotypical analysis with functional assays that assess their suppressive potential.

The suppressive potential of T_{regs} is best assessed with a suppression assay. For this assay, T_{regs} are isolated from a source of interest and subsequently co-cultured with activated conventional $CD4^+$ and $CD8^+$ T cells (responder cells) in several ratios. These responder cells are labeled with a fluorescent proliferation dye prior to the start of the co-culture. By analyzing the intensity of the proliferation dye after several days, one can assess to what extent T_{regs} from a source of interest can suppress responder cell proliferation (Figure 1). Although the focus of this six day protocol is on testing the suppressive potential of tumor-derived T_{regs}, the assay is applicable to murine T_{regs} obtained from virtually any tissue, disease model, or genetically manipulated context. In addition, the protocol allows modifications to fit the user's research question. This includes changing the type of responder cells, or supplementing the co-culture with blocking/stimulating agents, e.g. antibodies or cytokines of choice.

Satisfactory execution of a suppression assay can be quite tedious due to many different variables that have to be taken into account. For example, results can be influenced by the

chosen isolation method, choice of stimulatory signals and responder cells, and culture conditions. This chapter describes a protocol for the isolation of T_{regs} from murine mammary tumors, spleens and lymph nodes by flow cytometry-based cell sorting and analysis of their suppressive potential on splenic $CD4^+$ and $CD8^+$ T lymphocytes. We provide detailed descriptions of optimal sample preparation, sorting strategies, co-culture conditions, execution and analysis of the suppression assay.

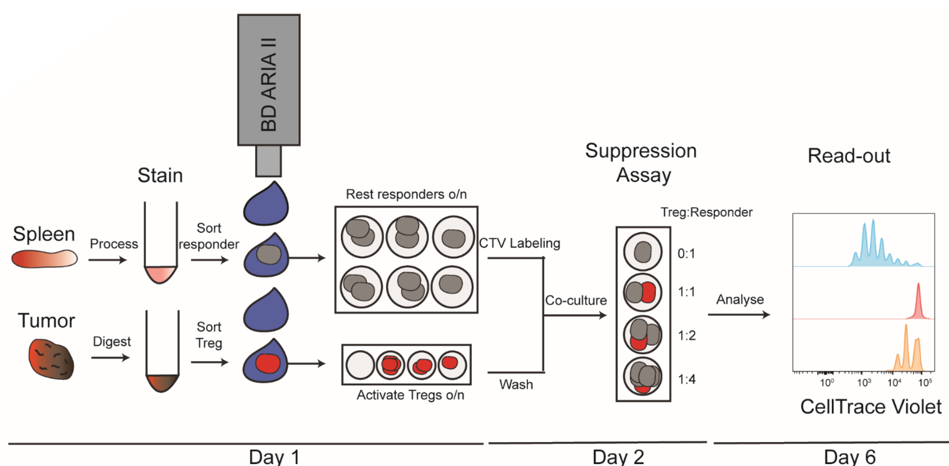

FIGURE 1. Schematic overview of the 6-day suppression assay described in this protocol.

EQUIPMENT AND MATERIALS

Preparation of single-cell suspensions from freshly isolated murine tissues

1. Freshly isolated murine tissues of interest in PBS on ice
2. McIlwain Tissue Chopper (Ted Pella Inc.) including chopping discs, blades and scalpel
3. Plunger of a 2mL syringe
4. Cell strainer, mesh size 70µm (Falcon)
5. Shaking waterbath or shaker
6. Red Blood Cell (RBC) lysis buffer: 155mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA in H_2O, pH 7.2–7.4 (Note 1).
7. Freshly prepared tumor digestion mix: 3mg/mL Collagenase A (Roche) in serum free DMEM (Dulbecco), Deoxyribonuclease I from bovine pancreas (DNAse) 25µg/mL (Sigma Aldrich)
8. Inactivation buffer: DMEM medium supplemented with 10% vol/vol FCS
9. Sorting buffer: 1x PBS, 0.5 % BSA, 2mM EDTA

Antibody staining & cell sorting

1. Freshly prepared single cell suspensions from tissues of interest on ice
2. Fluorescently labeled anti-mouse antibodies (Table 1)
3. LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermofisher)
4. 7-AAD Viability Staining Solution (Thermofisher)
5. Fc block: purified anti-mouse CD16/32 antibody (BD Biosciences)
6. 1.4ml Push Cap U-bottom tubes (Micronic)
7. 5mL polypropylene round-bottom tubes, 12x75mm (Falcon)
8. 5mL polypropylene tubes with 35µm cell strainer snap cap (Falcon)
9. Collection/Culture buffer: IMDM (Dulbecco) , 9% FCS, 100IU/mL penicillin, 100µg/mL streptomycin, β-mercapto-ethanol 0.2% (Merck), GlutaMAX 1% (Thermofisher)
10. A 3-laser flow cytometry based cell sorter, with the ability to detect at least 5 different fluorescent labels

TABLE 1. Recommended fluorochrome-conjugated antibody panel for optimized T_{reg}/responder cell sorting from murine tumor tissues.

Antigen	Fluorochrome	Clone	Concentration	Marker	Manufacturer
CD3	APC-eF780	145-2C11	1:400	T lymphocytes	BioLegend
CD4	PE	GK1.5	1:200	$CD4^{+}$ T lymphocytes	Thermofisher
CD25	APC	PC61	1:400	Regulatory T cells	Thermofisher
CD8	FITC	53-6.7	1:400	$CD8^{+}$ T lymphocytes	BD Biosciences

Suppression assay

1. Freshly sorted T_{regs} and responder cells on ice
2. Recombinant murine IL-2 (Peprotech)
3. Dynabeads™ Mouse T-Activator CD3/CD28 (Thermofisher)
4. CellTrace™ Violet Cell Proliferation dye (Thermofisher)
5. A 3-laser analytical flow cytometer, with the ability to detect at least 5 different fluorescent labels

Essential laboratory materials

1. 1x sterile phosphate buffered saline (PBS)
2. 12-multichannel pipette
3. Tissue culture-treated 96-well U-bottom plates (Greiner bio one)
4. Tissue culture-treated 6-well plates (Greiner bio one)
5. Sterile filter tips
6. 15 and 50mL polypropylene tubes (Falcon)
7. 1.5mL Eppendorf tubes (Eppendorf)
8. Refrigerated (plate) centrifuge

9. Cell counting equipment (manual/automated)
10. Ice bucket

PREPARATION OF SINGLE-CELL SUSPENSIONS FROM FRESHLY ISOLATED MURINE TISSUES

The suppression assay can be performed with T_{regs} obtained from any tissue, dependent on the research question of the experiment. It is important to realize that the number of T_{regs} that can be obtained will greatly vary between different tissues or stages of disease. T_{regs} can be quite easily isolated from spleen and lymph nodes and are therefore commonly used for standard assays. In contrast, murine tumors may harbor few intra-tumoral T cells, and accordingly yield a low number of T_{regs}. Isolating sufficient numbers of T_{regs} from tumors can therefore be challenging, but is essential for high quality results. T_{regs} and responder cells are isolated from single cell suspensions of murine tissues via flow cytometry based sorting. Typically, each murine tissue requires a specific approach for optimal preparation of single cell suspensions. In this protocol, we will describe the isolation of T_{regs} from murine spleen, lymph nodes and mammary tumors.

Keep cells sterile, on ice and protected from light, unless otherwise stated throughout the protocol. Before starting processing of tissues, cool PBS and sorting buffer on ice and warm the tumor digestion mix to 37°C (10mL in a 15mL tube per tumor)

Spleen and lymph nodes

1. Remove fat attached to the spleen/lymph nodes and place organ of interest on a (separate) 70µm cell strainer, inserted into a 50mL tube.
2. Gently mash the tissue with the plunger through the filter, while adding ~15mL cold sterile PBS until cells have passed through the cell strainer.
3. Centrifuge the cells at 300g, 4°C for 5 minutes and aspirate supernatant.
4. Incubate the spleen cell pellet (skip this step for the lymph node cells) in 1mL of sterile RBC lysis buffer for 2 minutes at room temperature. Stop RBC lysis with 10mL of cold sorting buffer and subsequently pellet the cells.
5. Resuspend the cells in 1mL (spleen) or 500 µL (lymph nodes) of sorting buffer on ice and count (manually/automated) the number of cells in suspension.

Mouse mammary tumors

The following protocol applies to tumors that measure up to a maximum of 1500mm^3 (Note 2).

1. Place the tumor on a chopping disc cleaned with 70% ethanol. Remove non-tumor tissue such as fat tissue. Make sure the tumor is free of lymph nodes, to prevent

contamination with lymph node-derived T_{regs}. Pre-cut the tumor in small pieces using a clean scalpel.

2. Place the disc under the tissue chopper and chop the tumor at least 3x, to a homogenous sample. Use maximum cutting speed and blade force, 5–10µm blade travel. Alternatively, other cutting instruments can be used to fragment the tumor.
3. Scrape the chopped tumor from the disc with a scalpel and carefully insert into a tube with 10ml of warmed digestion mix. Incubate at 37ºC for 1 hour with gentle agitation. This can be done in a shaking water bath, or with a shaker placed in an incubator.
4. After incubation, pass the cells using a plunger through a 70µm cell strainer capped into a 50mL tube and add 15mL of cooled inactivation buffer to stop the enzymatic digestion.
5. Centrifuge the cells at 300g, 4°C for 5 minutes and aspirate supernatant. Resuspend the cells in 1mL of sorting buffer on ice and count the cells.

The suppression assay requires the co-culture of T_{reg} and responder cells. Here, the responder cells are of a combination of splenic $CD4^{+}$ $CD25^{-}$ and $CD8^{+}$ T cells, which are co-cultured with T_{regs} in five different ratios for 96 hours.

In order to estimate the portion of the single cell suspensions that should be stained and sorted to obtain sufficient cells to perform the assay, it is important to calculate the required number of cells first. Testing a single condition in five T_{reg}:responder ratios requires a total input of 125.000 responder cells and 50.000 T_{regs}. Per ratio, 25.000 responder cells are plated. To obtain a T_{reg}:responder ratio of 1:1, 25.000 T_{regs} are co-cultured with 25.000 responder cells. The remaining 25.000 T_{regs} are serially diluted, with linearly fewer T_{regs} for each ratio. When testing T_{regs} from multiple conditions, multiply the number of required responder cells accordingly. This suppression assay has been optimized for an input of 50.000 T_{regs} but can be downscaled to an input of 10.000 T_{reg} cells.

As each tissue yields a different number of T_{regs}, we have summarized our typical sorting yields here. From a spleen of a naïve adult wild-type FVB mouse, $0.8*10^{6}$ $CD4^{+}$ $CD25^{+}$ T_{regs}, $3.0*10^{6}$ $CD8^{+}$ T cells and $10*10^{6}$ $CD4^{+}$ $CD25^{-}$ T cells can be recovered. The number of T_{regs} that can be obtained from a tumor is size and model dependent. From a tumor sized $1500mm^{3}$, we generally recover between 50.000 and 100.000 T_{regs}.

Estimate the portion of single cell suspension that should be fluorescently labeled and sorted accordingly, and proceed with the antibody staining procedure (Note 3).

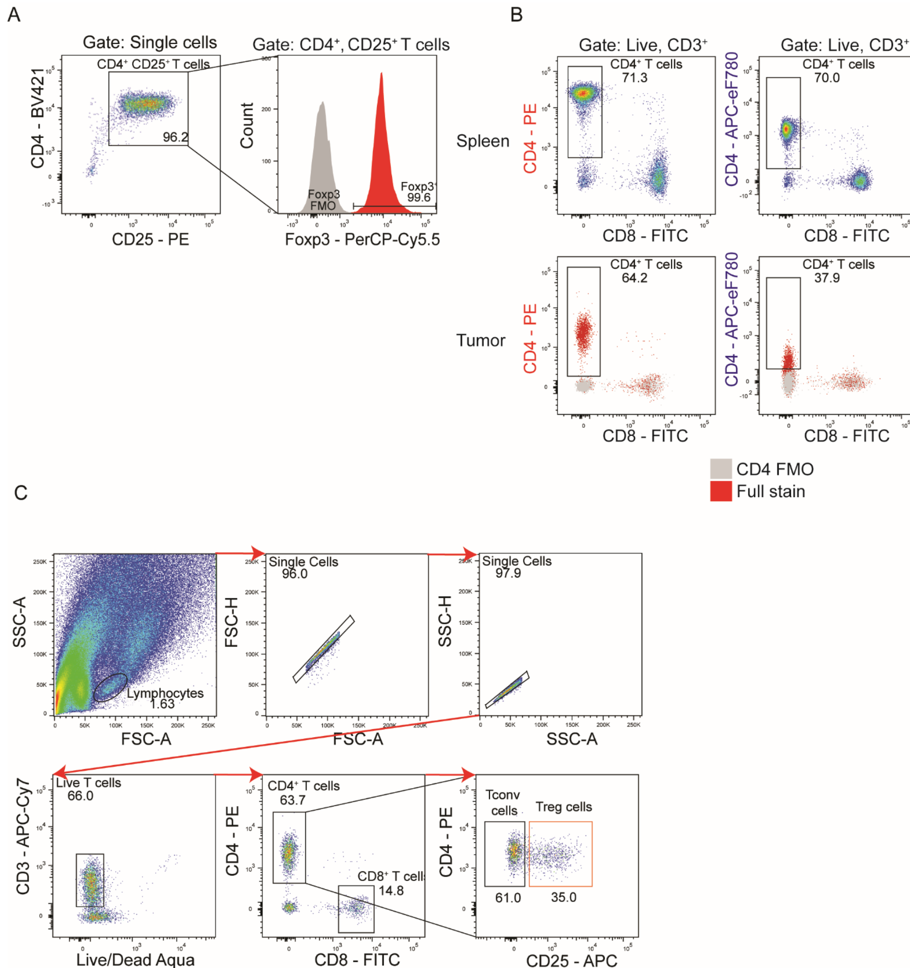

FIGURE 2. Flow cytometry-based sorting of regulatory T cells.
A. Splenic CD4+CD25+ cells were sorted as described and intracellularly stained for FOXP3 to confirm T_{reg} identity. **B**. Single cell suspensions from spleen and tumor were fluorescently labeled according to Table0 1, or an alternative panel using a relatively dim antibody for CD4. CD4 expression on live $CD3^+$ T cells is shown. **C.** Recommended gating strategy to sort T_{regs} from fluorescently labeled single cell suspensions. Tumor tissue is shown.

FLUORESCENT ANTIBODY STAINING & CELL SORTING

After the preparation of single cell suspensions, cells are fluorescently labeled in order to identify and sort T_{regs} and responder cells from the samples. Here, a thoroughly tested and optimized approach for the fluorescent staining and sorting of T_{regs} is presented. The choice of antibody-fluorochrome conjugate combination dictates the purity of the isolated T_{reg} population. $CD4^+$ T_{regs} in mice are characterized by high expression of the transcription

factor Foxp3. However, unless Foxp3-reporter mice are used, it is not possible to sort live T_{regs} based on this marker, because detection of intracellular Foxp3 requires fixation and permeabilization. To circumvent this, it is widely accepted to sort T_{regs} based on surface expression of CD4 and CD25 (IL2-receptor α-chain) [11]. Since CD25 can also be transiently expressed on conventional T cells upon TCR stimulation, it is important to confirm T_{reg} identity of sorted $CD4^{+}$ $CD25^{high}$ cells by intracellular Foxp3 staining, as shown in Figure 2A (Note 4). Additionally, tumor tissue-derived T_{regs} express relatively lower levels of CD4 as compared to T_{regs} derived from other tissues due to enzymatic treatment. Therefore, a relatively bright anti-CD4 fluorochrome-antibody conjugate must be used to discriminate these cells from the CD4 negative fraction, as illustrated in Figure 2B. Using the following protocol, T_{regs} can be directly sorted from the single cell suspensions without the need for additional purification steps.

Cell suspensions are first incubated with Fc receptor block solution (Note 5), followed by incubation with fluorescently labeled antibodies (Table 1). Finally, cells are stained with a viability dye to distinguish viable from dead cells. To compensate for spectral overlap, single fluorochrome stained samples should be prepared for each fluorochrome (Note 6). To determine positive populations for CD4-PE and CD25-APC, fluorescence minus one (FMO) controls should be included for each tissue that is sorted.

1. Plate the previously calculated number of cells from each single cell suspension in a 96-well plate. Plate a maximum of $6*10^6$ cells per well. Keep the plate on ice.
2. In the same 96-well plate, plate cells in 6 additional wells for each single fluorochrome sample and an unstained sample. For splenocytes, plate ~$1*10^6$ cells/well. When sorting tumor samples, plate compensation beads instead of cells to save sample for sorting.
3. Plate 2 extra wells for each tissue sample for CD4-PE and CD25-APC FMO controls. Plate same number of cells/well as determined in 3.1. The plate now contains cells for sorting, single fluorochrome controls, and FMO controls.
4. Prepare the following solutions in cold sterile sorting buffer (viability dye in PBS), 50µL per well.

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Solutions for fluorescent labeling of single cell suspensions

1. Antibody mix (Note 7)
    1. Prepare the mix using the antibodies and concentrations described in table 1. Prepare 50µL antibody mix per well. For example, if $60*10^6$ splenocytes are sorted, calculate for 10 wells. Prepare 50µL extra. (10 wells * 50µL) + 50µL = 550µL antibody mix.
2. Viability dye
    1. Fixable LIVE/DEAD Aqua cell stain kit (1:1000 in PBS)
3. Fc receptor block solution.

1. Dilute purified anti-mouse CD16/32 antibody (1:100)

Control solutions for flow-based sorting

4. CD4-PE and CD25-APC FMO controls.
   Prepare FMO controls for each tissue that will be sorted.
   1. Prepare the antibody mix as in a. but without the CD4-PE antibody.
   2. Prepare the antibody mix as in a. but without the CD25-APC antibody.
5. Single fluorochrome controls (Note 6)
   1. Prepare single fluorochrome controls by diluting each fluorochrome (including the viability dye) from table 1 separately. Use the indicated concentration.

5. Centrifuge the 96-well plate for 2 minutes at 380g after preparation of the solutions. Flick off the supernatant in a waste bin in the flow hood and gently press the plate upside down on a tissue.
6. Resuspend cell samples (all, except single fluorochrome controls) in 50µL of Fc receptor block solution and incubate 5 minutes on ice. After incubation, centrifuge the plate and discard supernatant.
7. During this incubation, add 50µL of single fluorochrome controls to designated wells.
8. Resuspend the fluorescent antibody mix and FMO controls and add 50µL to the designated wells. Gently mix with a pipette and incubate the plate for 20 minutes protected from light at 4°C.
9. After incubation, wash the plate 1x with PBS (Note 8)
10. Stain cells with viability dye according to manufacturer's instructions. After incubation, wash the cells 1x with sorting buffer.
11. Resuspend samples for single fluorochrome- and FMO controls in 200µL sorting buffer and collect in 1.4mL U-bottom tubes.
12. Resuspend each sample for sorting in 200µL sorting buffer. Filter the sample through a 35µm cell strainer into a 5mL polypropylene tube. Combine all samples from matching tissue in a single tube. Replace the cell strainer cap if clogged to enhance cell recovery. T_{regs}/responders will be sorted directly from this tube. Keep tubes sterile, on ice and protected from light.
13. Adjust sample volume to an appropriate cell concentration with sorting buffer, to increase sorting efficiency (Note 9). Guidelines:
    1. 70µm nozzle: $14*10^6$ - $21*10^6$ cells/mL
    2. 85µm nozzle: $10*10^6$ - $15*10^6$ cells/mL
    3. 100µm nozzle $4*10^6$ - $6*10^6$ cells/mL
14. Prepare 5mL polypropylene collection tubes with 1mL collection medium for collection of T_{reg}/responders (Note 10 & 11).
15. Sort cells in an aseptic fashion. Keep samples cooled at all times during the sorting process. Keep the collected cells on ice.
16. Apply the following gating strategy on single cells that are negative for the viability dye.

See Figure 2C for gating T_{regs} in tumor tissue (Note 12).

1. Regulatory T cells:
    1. $CD3^+$, $CD8^-$, $CD4^+$, $CD25^+$
2. SPLEEN RESPONDERS - Conventional $CD4^+$ T cells:
    1. $CD3^+$, $CD8^-$, $CD4^+$, $CD25^-$
3. SPLEEN RESPONDERS - $CD8^+$ T cells:
    1. $CD3^+$, $CD4^-$, $CD8^+$, $CD25^{+/-}$

17. Sort both $CD4^+$ and $CD8^+$ responder cells simultaneously in the same collection tube. (Note 13)
18. Sort T_{regs} from each tissue in a separate collection tube (Note 14).
19. Perform a purity check on collected cells after sorting (Note 15):
    1. Resuspend 20µL of the collected cell suspension in 180µL PBS
        1. Run ddH_2O at high differential pressure until sample line is clean
        2. Record 500 to 1.000 events in the gate of the sorted subset.
    2. Mix 20µL of collection buffer with 180µL PBS.
        1. Run ddH_2O at high differential pressure until sample line is clean
        2. Record for the same amount of time to determine background signal
    3. Determine the percentage of the population of interest from total events (Note 16).
20. Immediately proceed with the suppression assay. (Note 17)

SUPPRESSION ASSAY

A schematic representation of the assay is shown in Figure 3. Freshly sorted T_{regs} and responder cells are first cultured separately overnight to allow for activation and resting respectively. The following morning, responder cells are labeled with CellTrace™ Violet Proliferation (CTV) Dye (Note 18) and co-cultured with T_{regs} at different ratios for 96 hours. Finally, responder cell proliferation is analyzed by flow cytometry to determine the suppressive potential of the T_{regs}. This protocol is optimized for the analysis of low numbers of T_{regs}, and has been validated for an input as low as 10.000 T_{regs} per condition. However, if T_{reg} yield is sufficient, for robustness we strongly recommend performing the assay with 50.000 T_{regs}, and technical replicates for test conditions and controls.

Controls

In order to adequately determine how T_{regs} affect responder cell proliferation, it is essential to include appropriate controls during the co-culture of T_{regs} and responder cells.

1. To determine the maximum proliferative potential of responder cells in a given time period, responder cells should be cultured with stimulatory signals in the absence of T_{regs}. This is essential to verify that responder cells have the capacity to proliferate, allowing for a window of T_{reg} mediated inhibition of proliferation.

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2. To determine the background proliferative activity of unstimulated responder cells in a given time period, responder cells should be cultured in the absence of both T_{regs} and stimulatory signals. This is an essential control to verify that responder cells do not proliferate in the absence of stimulation. Additionally, the cells from this control are used to set up CTV signal for data acquisition.

FIGURE 3. Schematic plate layout for the suppression assay.
Follow stepwise instructions to co-culture T_{regs} and responder cells in five T_{reg}:Responder ratios in seven wells, per T_{reg} condition. Repeat for other conditions if necessary.

Additional considerations have to be taken into account in order to draw reliable conclusions from the assay. Although cell isolation by flow sorting allows for near 100% purity of the target populations, this method of cell sorting is performed under relatively high pressure conditions. As a result, shear stress may damage cells and alterations may occur in the redox state and metabolic profile [12]. This procedure may therefore directly impact the performance of cells in this assay. It is essential that after sorting, cells are first cultured separately overnight, to allow cells to recover from sorting induced stress as shown in Figure 4A (Note 19).

Another consideration is the method of responder cell stimulation, as there is a fine balance between excessive and inadequate stimulation. In this assay, responder T cells

are stimulated with anti-CD3 and anti-CD28 antibodies covalently bound to magnetic beads, leading to both TCR activation and co-stimulation. Although beads are simple and extremely reproducible in use, these beads do not fully mimic true antigen-presenting cells (APCs) and bypass any inhibitory effect on proliferation and priming mediated by T_{reg} signaling to APCs. If APCs are required, the user can exchange beads with APCs of choice. It is important to titrate bead:responder ratios because excessive stimulation of responder cells can lead to loss of T_{reg} mediated suppression, whereas inadequate activation may prevent responder cell proliferation. We have found that a ratio of bead:responder of 1:5 provides a window for suppression while simultaneously activating responder cells.

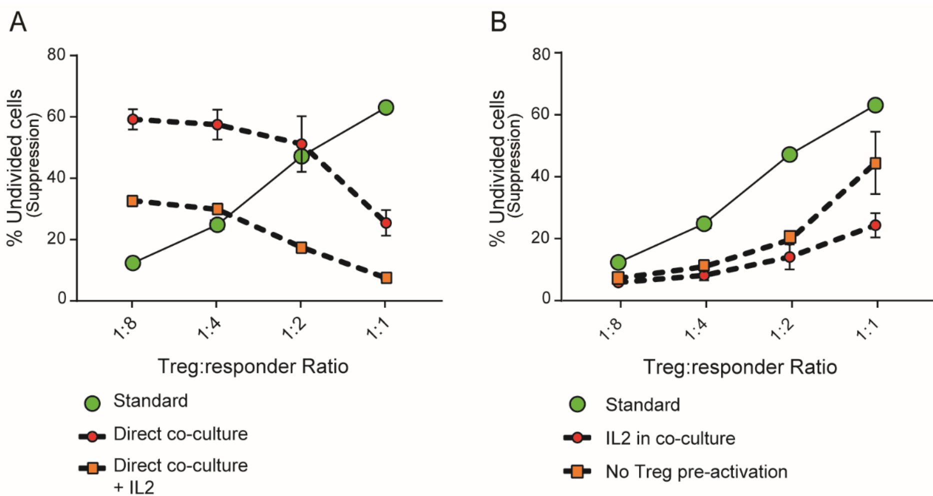

FIGURE 4. Impact of culture conditions on responder cell proliferation.
The percentage of undivided responder cells is plotted for T_{reg}:Responder ratios following suppression assays with various conditions. Standard data: Assay was performed as recommended, including overnight resting, resulting in ratio-dependent proliferation. **A.** When a resting period is omitted and cells are directly co-cultured, ratio-dependent proliferation is lost. **B.** Adding IL-2 (300U/mL) to the co-culture, or omitting T_{reg} pre-activation reduces assay sensitivity. Two technical replicates were used per condition, mean ± SEM is shown from two independent biological replicates.

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Additional to responder cell activation, overnight activation of T_{regs} prior to responder cell co-culture greatly enhances suppressive potential. T_{reg} pre-activation may be essential when sorting from homeostatic conditions (Figure 4B) (Note 20).

Finally, T cell proliferation protocols typically include supplementation of IL-2 to culture medium. However, supplementing the co-culture medium with IL-2 highly increases

responder cell proliferation in the presence of T_{regs}, thus severely reducing the window of T_{reg} suppression. It is therefore strongly recommended to perform the assay in the absence of IL-2. (Note 21) (Figure 4B)

Day 1 – T_{reg} pre-activation & Responder resting - See figure 3

Responder cells

1. Determine the number of responder T cells and centrifuge the cells for 5 minutes, 250g at 4°C. Discard supernatant and resuspend the cells in culture medium at a concentration of $1.0*10^6$ cells/mL. Do not add beads or recombinant IL-2.
2. Plate 2mL of the cell suspension per well in a tissue culture-treated 6-wells plate.
3. Incubate the cells overnight at 37°C, 5% CO_2.

T_{regs}

4. Accurately determine the number of T_{regs} and subsequently centrifuge the cells for 5 minutes, 250g at 4°C. Discard supernatant and resuspend the cells in culture medium at a concentration of $5.0*10^5$ cells/mL.
5. T_{reg} and responder cells will be co-cultured in the T_{reg}:responder ratios 1:1, 1:2, 1:4, 1:8 and 1:16. The following protocol applies to an input of 50.000 T_{regs} for each condition, which are all seeded into the "1:1" well.
6. Add CD3/CD28 coated Dynabeads™ in a 1:5 bead:cell ratio to the T_{reg} cell suspension and mix. (Note 22)
7. In a tissue culture-treated round bottom 96-wells plate, add 50 µL of culture medium to wells 4-7; add 100 µL of the T_{reg} cell suspension to well 3 (Figure 3).
8. Transfer 50µL of T_{reg} cell suspension to the well designated for the 1:2 ratio. Mix thoroughly, but avoid bubble formation. This will result in a two-fold dilution of both T_{regs} and beads.
9. Repeat step 8 in consecutive wells to serially dilute 1:4, 1:8 and 1:16 T_{reg}:responder ratios. Discard the leftover 50µL of T_{reg} suspension.
10. Finally, add 50µL of culture buffer + murine recombinant IL-2 (600U/mL) to all wells that now contain T_{regs}. Final volume per well is 100 µL.
11. Incubate the 96-well plate overnight at 37 °C, 5% CO_2.

Day 2 – T_{reg}:Responder co-culture

1. Harvest responder cells from the 6-well plate by thorough resuspension. Collect the cells in a 15mL tube and centrifuge the tube 5 minutes, 250g. Keep an aliquot of cells apart for step 4.3.2 (~25K cells)
2. Label responder cells with CellTrace™ Violet according to manufacturer's instructions.
3. After labeling, accurately determine the cell number. Recovery rate after sorting, overnight culture and labeling is typically ~40%.
4. Discard the supernatant and resuspend the responder cells at $1.25*10^5$ cells/mL in

culture medium.

5. Remove at least 200µL from the suspension ($2.5*10^4$ cells) and reserve for unstimulated controls.
6. Add CD3/CD28 coated Dynabeads™ in a 1:5 bead cell ratio to the responder cell suspension and mix.
7. Wash the 96-well plate containing the activated T_{regs} with culture medium.
8. Start T_{reg}:responder co-culture by adding 200µL of the responder cell suspension to all ratios and mix by pipette. Also add 200µL of responder cells to the stimulation control condition in well 2. Add cells from step 4.2.5 (unstimulated control condition) to well 1.
9. Incubate the cells at 37°C, 5% CO_2 for 96 hours (Note 23)

Day 6 - Analysis

The suppressive potential of T_{regs} will be determined by measuring the proliferation of responder cells on day 6 (Note 24). As both CD4 and CD8 T cells are used as responder cells, proliferation can be assessed for each cell type separately. Cells will again be fluorescently labeled according to Table 1, with the exception of LIVE/DEAD Aqua as this channel is now reserved for CTV. Instead of LIVE/DEAD Aqua, we recommend using 7-AAD viability staining solution to detect dead cells (Note 25).

1. After incubation, wash cells 1x in sorting buffer
2. Re-stain cells according to step 3.2-3.10, omit FMO controls. Use beads for single fluorochrome controls.
    1. A small sample of the stimulated control cells can be used as a single fluorochrome control for CTV.
    2. A small sample of unstained stimulated control cells are spiked into the CTV sample to obtain a negative population.
    3. A small sample of unstained stimulated control cells are stained with 7-AAD
3. Resuspend cells in 100µL sorting buffer + 7-AAD (1:100) and proceed with cell analysis on a flow cytometer.
4. Prior to compensation, acquire an unstimulated responder cell sample and adjust detector gain of the channel used for CTV. Set the signal of the undivided peak to an intensity of 10^5.
5. Run compensation controls and set up gates. Gate Live, single, $CD3^+$ CTV^+ cells to exclude T_{regs} from the analysis (Note 26).

Record sufficient CTV^+ cells to perform descriptive and analytical statistics with the predetermined level of confidence. Ideally, a minimum number of 2.000 CTV^+ events is recorded.

Data analysis

To assess the suppressive potential of T_{regs}, perform fluorescence compensation on recorded

samples and gate: Single, Live, CD3+, CTV+ cells. The first peak (intensity 10^5) indicates the undivided population. Each subsequent peak indicates consecutive cell division. The CTV- fraction represents the T_{regs}. At this point, it is not possible to discriminate T_{regs} from responder cells based on CD25 expression, as responder cells will have upregulated CD25 in response to strong TCR stimulation. Confirm stimulation-dependent proliferation in controls. Draw gates to determine the percentage of divided/undivided fraction of the cells (Figure 5). For each sample, determine the percentage of the undivided population, and plot this according to T_{reg}:responder ratios (See graph type in Figure 4). The impact of T_{regs} from different conditions on responder cell proliferation can now be assessed (Note 27). An appropriate statistical test can be applied using the mean of the variable "% of undivided cells".

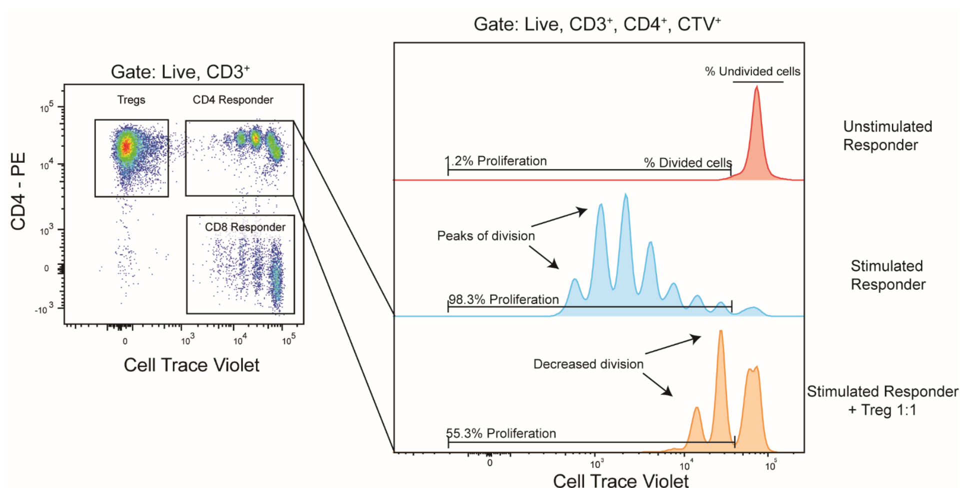

FIGURE 5. Recommended analysis of the suppression assay.
A suppression assay was performed as described with splenic T_{regs} and analyzed 96 hours after co-culture (day 6 of the assay). Responder cells do not proliferate in the absence of stimulation, but strongly divide when stimulated. T_{regs} potently suppress responder cell proliferation. T_{reg}:Responder ratio of 1:1 is shown.

NOTES

1. Commercially available RBC lysis buffers can contain fixation solutions. These should be avoided to maintain viability and cell function.
2. Mechanical disruption and enzymatic digestion is used to dissociate tumor tissue into a single cell suspension. Both treatments can result in cell damage and subsequent cell death. Additionally, enzymatic digestion can lead to a reduction of the available recognizable epitopes of cell surface markers. Preparation of single cell suspensions from tumor tissue should be optimized, *i.e.* duration and force of mechanical disruption,

enzyme choice, concentration and incubation time should be determined empirically per tumor type[13,14]. The described digestion method is optimized for mammary tumors[15].

3. The abundance of T_{regs} varies per tissue type. To sort sufficient cells to perform the assay, it is important to fluorescently label an adequate proportion of single cell suspensions. For tumor samples, we recommend to use 100% of the available tissue for sorting. For responder cells, sort at least 2-3 times the required number of cells to compensate for cells lost during washing and labeling steps.
4. Contamination of activated T-helper cells within the $CD25^{high}$ T_{reg} population may depend on mouse strain, genotype, tissue and homeostatic conditions of mice within the animal housing facility.
5. Murine myeloid- and B cells have high expression of Fc receptors, that specifically bind the Fc domain of fluorescently labeled antibodies. This may lead to false interpretation of positive signal, and could lead to sorting of a contaminated population. Therefore, incubate single cell suspensions in αCD16/32 Fc blocking reagent.
6. Guidelines for single fluorochrome controls
    1. The compensation matrix is determined with an algorithm that is embedded in the cell sorter software. To calculate the compensation values, the algorithm requires an accurate determination of the median fluorescent intensities (MFI) from the negative population and positive fluorochrome carrier population for each channel. An accurate estimate of the median is dependent on the variance, and the minimal number of negative and positive events required is between 2.000 and 5.000 for each.
    2. The median fluorescent intensity of the positive population must be at least as high as the highest median fluorescent intensity of an experimental sample. It is recommended to perform compensation on cells obtained from tissue that will be used for sorting T_{regs}. When sample is limited, the use of antibody-binding beads is recommended.
    3. The median fluorescent intensities of the negative and positive populations must be within the linear range of the detectors that are used.
    4. The antibody-fluorochrome conjugates used to prepare the single stains must be the same as those used in the experimental samples.
7. When the recommended antibody panel is modified, we advise to titrate antibody concentrations to determine the stain index and effect on data spread in secondary channels. The goal is to maintain optimal discrimination between negative and positive cell populations.
8. Washing entails the process of centrifuging the plate for 2 minutes at 380g, subsequently flicking off the supernatant and resuspending all samples in PBS followed by centrifugation. Lastly, flick of the supernatant and continue.
9. Nozzle diameter is determined as follows: average diameter of cells of interest * 6. For lymphocytes, the 70µm nozzle is acceptable. It also allows for acceptable duration of the sort. Using larger nozzles results in higher recovery and increased cell function, but

samples may require pre-enrichment to ensure acceptable sort times.

10. Sorting charged droplets that contain cells of interest can lead to accumulation of electrostatic charge inside a tube made of insulating polymers. Therefore, polypropylene tubes should be used to avoid build-up of electrostatic charge. Additionally, sorting into polypropylene tubes results in lower cell adherence to the tube walls, as compared to polystyrene tubes.
11. Addition of at least 1-2% FCS to the collection buffer has been shown to strongly reduce negative effects of cell sorting on cell viability, redox and metabolic perturbations. [12,16]
12. $CD8^+$ and $CD4^+$ T cell proliferation can be mitigated by multiple cell types, especially in the tumor microenvironment. To investigate the specific role of regulatory T cells on the suppression of cytotoxic T cells, the isolated T_{reg} fraction must be free from contamination of other cell types. In this specific assay, CD3 is used to uniquely identify cells of interest, and exclude other cell types that may bias the results. For example, DCs and B lymphocytes.
13. Sorting both CD4 and CD8 responder cells allows for investigating T cell-specific suppression. In this assay, cells are sorted in physiological ratios (CD4:CD8 = ~3:1 in spleen), but this can be adjusted to the user's preference, including sorting CD4 and CD8 T cells separately. Investigating suppression of CD4 and CD8 T cells provides a general method of assessing suppression of T_{regs}. Alternatively, it is possible to use virtually any other cell type of interest as responders.
14. Sorting T_{regs} and responder cells from spleen samples can be performed relatively fast, and takes approximately ~20-30 minutes per conditions in our experience. In contrast, sorting tumor samples can take up to multiple hours due to high cell concentration and a low T_{reg} fraction. In case of insufficient T_{reg} yield from tumors, an optional pre-enrichment of tumor samples may be performed. Sterile isolation of $CD45^+$ cells from the tumor sample via positive magnetic selection may increase yield and does not interfere with surface staining. Alternatively, percoll gradient separation may increase yield, although we have not tested this in this assay.
15. Doublets with $CD3^+$ cells and incorrect determination of charge delay timing (drop delay) may result in contamination or insufficient isolation of the cells of interest. A post-sort (purity) check can be performed to evaluate the collected samples.
16. The median fluorescent intensity may be slightly lower after sorting. Adjustment of the gates may be required to estimate to percentage correctly.
17. In our experience, storing cells on ice for up to one hour after sorting does not impact cell viability.
18. CellTrace™ Violet, is a fluorescent dye which covalently binds free amines on the surface and the inside of cells. When cells divide, the dye is equally divided over daughter cells, resulting in a 50% reduction of signal intensity per division. This allows for the visualization of proliferation in the responder cell pool. Alternatively, responder cells can be labeled with fluorescent proliferation dyes such as CFSE. This does require

modification of recommended antibody panels.

19. For cell sorting, we used a FACSAria II (BD Biosciences) with a 70µm nozzle and 70psi. Likely, the observed negative effect on proliferation when cells are co-cultured directly after cell sorting is due to shear stress from the cuvette-based cell sorter. If a resting period is undesired, this effect may be minimized by using a jet-in-air based cell sorter at lower sheath pressure, e.g. with a 100µm nozzle and 20psi. When omitting sorting-induced stress is not required (If using magnetic-based cell isolation for example), alternative assays exist as discussed here[17].
20. In our experience, T_{reg} pre-activation does not impact pre-existing differences in suppressive potential between T_{reg} populations.
21. Conventional CD4 T cells produce high levels of IL-2, whereas T_{regs} do not, although essential for survival of both. Accordingly, a proposed mechanism of T_{reg} suppression is CD25-mediated depletion of IL-2 from the local microenvironment[18].
22. Beads added for T_{reg} activation on day 0 will not induce responder cell proliferation during co-culture, for which additional beads are added.
23. Co-culture time may be adjusted as preferred, but should be minimally 72 hours.
24. Further in-depth analysis may include flow-cytometric analysis of responder cells and T_{regs} focused on the expression of cell surface receptors, intracellular cytokine production and cell-death. Culture medium can be collected after co-culture for cytokine analysis.
25. For analysis, an LSR II SORP (BD Biosciences) was used. The configurations are provided in table 2.
26. Divided $CD4^+$ responder cells may lose CTV to such an extent that these cells become indistinguishable from T_{regs} during analysis. In case of prolonged co-culture, it is advisable to additionally label the T_{reg} cells. This could be done by using alternative fluorescent proliferation labels, congenic markers, or including Foxp3 in the analysis panel.
27. Additional methods for analyzing T cell proliferation that may provide higher sensitivity are reviewed here[19]. Alternatively, specialized software to analyse proliferation data can be used (ModFit LT™, Flowjo LLC).

TABLE 2. LSR II Configurations for suppression assay analysis.

Antigen	Fluorochrome	Configuration FACSAria II	Configuration LSR II SORP
CD3	APC-Cy7	633nm laser (20mW); 750LP, 780/60	638nm laser (40mW); 750LP, 780/60
CD4	PE	488nm laser (20mW); 565LP, 585/42	561nm laser (40mW); 565LP, 585/42
CD8	FITC	488nm laser (20mW); 502LP, 530/30	488nm laser (50mW); 505LP, 525/50
CD25	APC	633nm laser (20mW); 660/20	638nm laser (40mW); 670/14
Free amines	L/D Aqua	405nm laser (25mW); 502LP, 530/30	
Free amines	CTV		405nm laser (40mW); 450/50
dsDNA	7-AAD		561nm laser (40mW); 600LP, 610/20

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