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In Vivo Treg Suppression Assays

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Abstract

To fully examine the functionality of a regulatory T cell (T_{reg}) population, one needs to assess their ability to suppress in a variety of in vivo models. We describe five in vivo models that examine the suppressive capacity of T_{regs} upon different target cell types. The advantages and disadvantages of each model including resources, time, and technical expertise required to execute each model are also described.

Keywords

T_{reg} ; Homeostasis; IBD; Experimental colitis; EAE; Tumor; B16 melanoma; In vivo; Foxp3

1. Introduction

The suppressive activity of regulatory T cells (T_{regs}) is most conveniently assessed using standard in vitro T_{reg} assays (see Chapter 2). Although performing these assays is an important step in deciphering the function of a regulatory population, in vitro culture conditions cannot replicate the complex in vivo microenvironment. Consequently, assessing T_{reg} function in vivo is more physiologically relevant. Indeed, in vivo assays provide a more significant regulatory challenge for T_{regs} than in vitro assays. For instance, IL10-deficient T_{regs} are fully functional in vitro but defective in a variety of in vivo models (1–3). Despite the importance of in vivo assays to assess T_{reg} function, they are clearly more technically challenging as they tend to require time to complete, more resources, and often more T_{regs} than in vitro assays. However, in vivo T_{reg} suppression assays represent an important tool in assessing the function of this critical immune population.

Here, we describe five different in vivo models that assess T_{reg} function: (1) homeostasis model, (2) inflammatory bowel disease (IBD) recovery model, (3) experimental autoimmune encephalomyelitis (EAE) model, (4) B16 melanoma model, and (5) *Foxp3*[−] rescue model. These models are very effective at elucidating T_{reg} function while only requiring between 0.5 and 1×10^6 per T_{regs} mouse. The requirements and pros and cons of the five models are illustrated in Table 1. We would recommend the use of at least three in vivo models to assess the regulatory activity of a test population, although additional models would clearly provide a more detailed examination. It should be noted that this is not intended to be an exhaustive list, but rather a collection of methods that function in vivo. Other have been frequently used to assess T_{reg} models have been described, but many are less well-characterized (4–6).

³²Caution: Irritant and suspected carcinogen. Refer to the manufacturer's MSDS for more details.

2. Materials

2.1. Common to all Protocols

1. All of the models require mice for donor T cell populations as well as *Rag1*^{-/-} or *Foxp3*^{-/-} mice for recipients. The number of mice required differs depending upon the model, the number of experimental groups, and the number of replicate experiments.
2. Blocking solution: 10% sterile mouse serum in PBS + 5% FBS.
3. Murine cell culture medium: RPMI [Mediatech] supplemented with 10% FBS [optimal manufacturer and lot to be determined empirically], 2 mM l-glutamine [Mediatech], 1 mM Sodium Pyruvate [Mediatech], 100 mM Non-Essential Amino Acids [Mediatech], 5 mM HEPES free acid [Mediatech], 10 ml of 5.5×10^{-2} 2-mercaptoethanol [Invitrogen], and 100 U/ml Penicillin/Streptomycin [Mediatech] (see Note 1).
4. Gey's solution for red blood cell lysis: 12 mM potassium bicarbonate 156 mM ammonium chloride (KHCO₃), (NH₄Cl), diluted in water. Filter sterilize the solution through a 0.2-μm filter.
5. V-bottom 96-well tissue culture plate [Nunc].
6. 70 μM nylon cell strainer [Beckton Dickinson].
7. 50 ml conical tubes.
8. 15 ml conical tubes.
9. Sterile normal mouse serum [Gibco].
10. Phosphate buffered saline (PBS) [Mediatech].
11. Hanks balanced salt solution (HBSS) [Mediatech].
12. Sterile 1 ml syringes, use plunger for homogenization [Beckton Dickinson].
13. Sterile 3 ml syringes [Beckton Dickinson].
14. Sterile 27G needles [Beckton Dickinson].
15. Fluorescently tagged antibodies (CD4, CD25, CD45RB, Thy1.1, Thy1.2, Foxp3).
16. 40 μM nylon cell strainer [Beckton Dickinson].
17. Fluorescent activated cell sorter (FACS) buffer: PBS + 0.05% + 5% FBS. NaN₃
18. Trypan Blue.
19. Scissors and forceps suitable for tissue collection.
20. 24-well cell culture plate [Corning].

2.2. IBD Model

1. Sterile 23G needles [Beckton Dickinson].
2. Sterile 10 ml syringes [Beckton Dickinson].
3. Digital weighing scale.

¹The optimal manufacturer and lot number of FBS/FCS can vary; therefore, this must be determined empirically. Prior to use in assays, FBS must be heat inactivated for 30 min at 56°C. Following heat inactivation, FBS can be stored at 4°C for up to 1 month.

4. Plastic container such as a pipette tip box lid (Not absolutely required but useful as a reference for accurate weight measurement and also used to place the mouse while weighing).
5. Tissue cassettes for histology [ThermoFisher Scientific].
6. 10% Neutral buffered formalin solution [ThermoFisher Scientific].

2.3. EAE Model

1. Incomplete Freund's adjuvant (IFA) [ThermoFisher Scientific].
2. *Mycobacterium tuberculosis* H37Ra (killed and desiccated) [ThermoFisher Scientific] (see Note 2).
3. Solution of MOG35–55 (MEVGWYRSPFSRVVHLYRNGK) peptide diluted to 1 mg/ml in PBS.
4. *Bordetella pertussis* toxin, diluted to 1 µg/ml in PBS [ThermoFisher Scientific] (see Note 3).
5. Two 2-ml glass Hamilton syringes with double-ended locking hub (Luer-lock) connector or 3-way stopcock [ThermoFisher Scientific].
6. Sterile 1 ml tuberculin slip tip syringes [Beckton Dickinson].
7. Sterile 25G needles [Beckton Dickinson].
8. Isofluorane anesthesia apparatus (optional).
9. Mouse ear clipper.
10. Frosted glass tissue homogenizer [ThermoFisher Scientific].
11. Percoll [Amersham Bioscience].

2.4. B16 Melanoma Model

1. Sterile blunt needles.
2. Sterile 30G needles [Beckton Dickinson].
3. B16 culture media: RPMI [Mediatech] supplemented with 7.5% FBS [optimal manufacturer and lot to be determined empirically], 2 mM l-glutamine [Mediatech], 100 mM Non-Essential Amino Acids [Mediatech], and 100 U/ml Penicillin/Streptomycin [Mediatech].
4. T175 flasks [ThermoFisher Scientific].
5. Trypsin-EDTA [Mediatech].
6. Isofluorane anesthesia apparatus.
7. Heating pad or heat lamp.
8. Dial caliper [Bel-Art Products].
9. RPMI media without any additives [Mediatech].
10. 2 ml cryo vials [Nunc].

²Caution: *Mycobacterium tuberculosis* is an inflammatory reagent. Avoid inhalation, skin or eye exposure. Use gloves, protective eyewear, and a mask when handling the reagent. Refer to the manufacturer's MSDS for more details.

³Caution: *Bordetella pertussis* toxin is a bacterial virulence factor. Avoid skin or eye exposure, or inhalation. Use gloves, protective eyewear, and a mask when handling the reagent. Refer to the manufacturer's MSDS for more details.

11. Small electric razor [Oster].
12. Q-tips.
13. Surgical providone iodine solution [Appicare Inc.].
14. Single use alcohol pads [ThermoFisher Scientific].
15. Blunt forceps [ThermoFisher Scientific].
16. Surgical scissors [Roboz].
17. Neosporin triple antibiotic ointment [ThermoFisher Scientific].
18. Buprenorphine or Rimadyl [must be obtained through a pharmacy].
19. Steel wound clips and Autoclip wound clip applicator [Beckton Dickinson].
20. Autoclip wound clip remover [Beckton Dickinson].
21. Percoll [Amersham Bioscience].
22. 5% H₂O₂ in PBS.

2.5. Foxp3⁺ Rescue Model

1. Insulin syringe fitted with a 30-G needle [Beckton Dickinson].
2. Camera.
3. Ruler or other scale bar.
4. Soft tissue organ cassettes [ThermoFisher Scientific].
5. 24-well cell culture plate [Corning].
6. Tissue cassettes for histology [ThermoFisher Scientific].
7. 10% Neutral buffered formalin solution [ThermoFisher Scientific].

3. Methods

3.1. Purification of Mouse T_{conv}/T_{reg} for In Vivo T_{reg} Suppression Assays

Mouse T_{conv} and T_{reg} can be separated using fluorescently conjugated antibodies, based on their expression of cell surface can be separated using only CD4 proteins. Mouse T_{conv} and T_{reg} and CD25 markers. However, by also staining with CD45RB, naïve T_{conv} can be separated from memory T_{conv} and T_{reg}, resulting in better purity of both populations. A similar strategy can be utilized by staining cells with CD44 and CD62L, where CD44^{low}/CD62L^{high} populations represent the naïve, T_{conv} cells. To maximize purity and recovery, one would ideally utilize a Foxp3 reporter strain, such as *Foxp3*^{GFP} (7), crossed with the mutant strain of interest.

Fluorescence activated cell sorting (FACS) is the preferred method of cell purification because of the purity of cell populations obtained. Greater than 95% purity can routinely be obtained by FACS. If FACS is not possible or available, an alternative method of purification utilizes antibodies coupled with magnetic or paramagnetic particles for cell sorting. Cells should be prepared using the manufacturer's guidelines (e.g., MACS - http://www.miltenyibiotec.com/en/NN_21_MACS_Cell_Separation.aspx, Dynabeads - [http://tools.invitrogen.com/content/sfs/manuals/114%2063D.Dynabeads%20FlowComp%20Mouse%20CD4%20CD25%20Treg%20Cells\(rev001\).pdf](http://tools.invitrogen.com/content/sfs/manuals/114%2063D.Dynabeads%20FlowComp%20Mouse%20CD4%20CD25%20Treg%20Cells(rev001).pdf)). Under optimal conditions, one can obtain purities of 85–90% by MACS. If an induced regulatory population is being assessed, methods appropriate for their generation and purification should be used. These

methods are also detailed in the companion Chapter 2. Additionally, it is advisable to enrich for T cells prior to sorting to reduce the amount of sorting time required. T cell enrichment can be done by removing the B cells by a standard panning protocol, Dynabeads or by MACS (see Note 4). Regardless of the purification method used, it is imperative that the purity of all sorted populations are confirmed by flow cytometry prior to commencing in vivo assays.

1. Harvest spleen and lymph nodes from mice.
2. Tease apart tissue with the plunger from a 1-ml syringe through a 70- μ m cell strainer into a 50-ml conical tube. Rinse strainer twice with HBSS to recover all cells. Alternatively, splenocytes may be teased apart between two frosted glass microscope slides.
3. Centrifuge homogenate at $300 \times g$ (1200 rpm) for 10 min.
4. Resuspend homogenate in 1 ml Gey's solution per spleen. Gently swirl for 2 min and then quench reaction by adding 12 ml of HBSS.
5. Centrifuge at $300 \times g$ for 10 min (see Note 4).
6. Resuspend cells in blocking solution at 0.5 ml per spleen.
7. Incubate cells for 10 min at 4°C.
8. Add fluorescently conjugated antibodies at a final concentration of 1:200 at 0.5 ml per spleen for 20–30 min at 4°C. For example, anti-CD4 Alexa 647 (or APC), anti-CD45RB (PE), and anti-CD25 FITC (see Note 5).
9. Wash cells with 5 ml PBS + 5% FBS. Centrifuge cells at $300 \times g$ for 10 min.
10. Resuspend cells in PBS + 5% FBS and strain through 40 μ m filter.
11. Purify cells by FACS according to the profile shown in Fig. 1.
12. Determine the purity of the sorted cells by flow cytometry.

In some cases, it is desirable to expand T_{regs} to generate greater cell numbers. Murine T_{regs} can be expanded using the following protocol: Murine T_{regs} are activated at 5×10^5 cells/ml in a 96-well round bottom plate in complete RPMI medium containing 1 ng/ml PMA, 200 ng/ml Ionomycin, and 100 IU/ml murine IL-2. Following 4–5 days of activation, cells should be washed and resuspended in culture media containing 50 IU/ml IL-2 at a density of 5×10^5 /ml in a 24-well culture plate. Cells can be maintained in IL-2 supplemented media and passaged to maintain a cell density of 5×10^5 cells/ml. Following 10 days in culture, T_{reg} expansion is approximately tenfold. Expanded T_{regs} maintain Foxp3 expression and suppressive capacity.

3.2. Statistical Analysis of Results

In all the models, it is important to determine the statistical significance between groups. A variety of statistical methods can be used. When comparing two independent samples of continuous data, a two-sample *t*-test is recommended when the normality assumption is reasonable. If the data are heavily skewed, contain outliers or the normality assumption is not valid for any reason, the Wilcoxon-Mann-Whitney test is the preferred nonparametric

⁴Panning is an economical method for B cell depletion and will reduce the sort time required. Panning is done following RBC lysis (step 5). The cells are incubated on sterile nontissue culture treated plates coated with goat antimouse Ig. Thus, the B cells will bind to the plate and the nonadherent cells (predominantly T cells) are collected and stained for sorting.

⁵Antibody conjugates can be altered depending upon laser availability. Optimal antibody concentrations must be determined empirically

alternative. Three or more independent groups should be compared using one-way ANOVA or a nonparametric analysis such as the Kruskal-Wallis test. Two related samples (paired) should be compared using the paired t-test or the Wilcoxon signed rank test. In all parametric analyses, means should be reported with a 95% confidence interval or the standard error. Results from non-parametric analyses should include the median, minimum, and maximum. *P*-values should be reported in all cases. In the experiments that require analyses at certain points over time such as EAE disease progression, weight change over time in the IBD model, and kinetics of tumor growth in the B16 melanoma model, more advanced statistical analyses are required because of the correlation between the data points. Therefore, the type and number of statistical analyses should be determined empirically.

3.3. Homeostasis Model

This model assesses the ability of T_{regs} to suppress the homeostatic expansion of T_{conv} cells upon transfer into a lymphopenic *Rag1*^{-/-} host. In this model, T_{conv} cells are sorted from B6.PL mice that are sorted from express the congenic marker Thy1.1, and the T_{regs} are sorted from C57BL/6 mice that express Thy1.2. The T_{conv} cells (Thy1.1) are transferred alone or with T_{regs} (Thy1.2) into *Rag1*^{-/-} mice. Seven days later, the number of T_{conv} cells is determined in the spleens of the recipient mice. Typically, there is a 50% reduction in the number T_{conv} when they are transferred with T_{regs} (8,9). The CD4⁺ T cells that are controlled by T_{regs} have a memory-like phenotype but are otherwise naïve and are not activated (10,11). Thus, this model assesses the capacity of the test T_{reg} population to control homeostatically expanding “naïve” T cells.

3.3.1. Injection of T_{conv} and T_{regs}

1. Sort T_{regs} and T_{conv} cells from mice with different congenic markers as described in Subheading 3.1. It is advisable to use different congenic strains to distinguish T_{reg} from the T_{conv} cells during analysis. This protocol describes the use of B6.PL mice (mice that express the congenic marker Thy1.1) for the isolation of the T_{conv} . However, B6.SJL-*Ptpca Pep3b*/BoyJ mice, which express the congenic marker, CD45.1, as opposed to CD45.2 (expressed on cells from C57BL/6 mice) can also be used.
2. Following the sort, centrifuge cells at $300 \times g$ (1,200 rpm) for 10 min. Resuspend the T_{regs} in 1 ml of PBS + 0.1% FBS and the T_{conv} cells in 2 ml of PBS + 2% FBS.
3. Count the cells using a hemocytometer and trypan blue to exclude dead cells
4. Dilute the T_{regs} to 5×10^5 cells/ml and the T_{conv} to 2×10^6 cells/ml with PBS + 0.1% FBS.
5. Determine the number of *Rag1*^{-/-} recipient mice that will be used per group based upon the total number of T_{regs} and T_{conv} (see Note 6).
6. Use one 15 or 50-ml conical tube per group and add the following: for T_{conv} only group add 1 ml of T_{conv} cells per mouse in the group (e.g., 5 mice = 5 ml of T_{conv}), for the T_{conv} plus Treg groups add 1-ml each of T_{regs} and T_{conv} per recipient mouse in the group and vortex cells (e.g., 5 mice = 5 ml of T_{conv} + 5 ml of T_{regs}) (see Note 7).

⁶Typically, there will be three groups of recipient mice: T_{conv} only, T_{conv} + “control” T_{regs} , and T_{conv} + “Experimental” T_{regs} . Ideally, it is important to have at least three mice receive T_{conv} cells only as this group is the control group to which groups will be compared. Each mouse will the T_{conv} + T_{regs} receive either 2×10^6 T_{conv} or 2×10^6 T_{conv} + 0.5×10^6 “control” or “Experimental” T_{regs} . Occasionally, a T_{reg} only group is included, especially if molecules regulating T_{reg} homeostatic control are being studied.

7. Centrifuge cells for $300 \times g$ for 5 min.
8. Resuspend cells in X ml of PBS + 0.1% FBS (where X = the number of mice in the group multiplied by 0.5 ml) (see Note 8).
9. Load the cells into a 3-ml syringe and inject *Rag1*^{-/-} mice intravenous (i.v.) into the tail vein with 0.5 ml/mouse using a 27-G needle (see Note 9).

3.3.2. Analysis of Experimental Mice

1. Seven days later euthanize mice, dissect spleens and place into separate labeled tubes of HBSS (see Note 10).
2. Process the individual spleens as detailed in Subheading 3.1.
3. Resuspend cells in 1 ml of RPMI + 10% FBS.
4. Count the cells using a hemocytometer and trypan blue to exclude dead cells
5. Stain 200 μ l of cells first with 10% normal mouse sera in FACS buffer for 5 min on ice to block the Fc receptors (see Note 11) and then CD4 and the appropriate congenic markers (e.g., Thy1.1 [distinguish T_{conv}] and Thy1.2 [distinguish T_{regs}]) in a 96-well V-bottom plate for 20 min on ice.
6. Centrifuge cells at $300 \times g$ for 2 min and wash twice with FACS buffer.
7. Resuspend cells in 100 μ l of FACS buffer and analyze by flow cytometry to determine the percentages of T_{regs} (Thy1.2⁺ cells) and T_{conv} (Thy1.1⁺ cells).
8. Calculate the number of T_{conv} and/or T_{reg} cells per recipient by multiplying the total number of live cells in the spleen by the percentage of T_{conv} and T_{regs}.

3.4. Inflammatory Bowel Disease (IBD) Recovery Model

The mucosal surface of the intestine is exposed to a variety of antigenic insults from dietary intake and the commensal flora. Regulatory T cells are important in maintaining intestinal homeostasis and preventing inflammatory bowel disease (IBD) in both humans and mice (12,13). Experimental colitis in mice closely mimics many of the symptoms of human IBD and is a very useful model to assess the function of T_{regs} in a mucosal environment.

Experimental colitis is induced by transfer of naïve CD4⁺CD45RB^{high} T cells into immunodeficient mice resulting in following wasting disease within 4–6 weeks (14). Injection of T_{regs} the onset of disease symptoms leads to recovery from the disease (15). In this murine model of IBD, the disease is induced by the expansion of autoreactive T cells in combination with antigenic factors present in the intestinal flora. One example is *Helicobacter hepaticus*, which is a common pathogen found in many mouse facilities. This pathogen normally colonizes the cecum and colon and causes disease in susceptible hosts (16). Our laboratory has adopted this recovery model of colitis as it provides a robust method for assessing T_{reg} function. As an alternative, some labs are injected at the use a

⁷It is important to add the cells in 1 ml aliquots and to vortex very well prior to centrifugation to ensure that all samples are treated equally and equivalent cell numbers are achieved in all groups. Deviation from this may result in variability in cell numbers during analysis.

⁸For example, if you are injecting four mice, resuspend in 2.0 ml. Also, to account for loss due to cell transfers, etc., it is advisable to add an additional 10% by volume of PBS + 0.1% FBS.

⁹It is critical to ensure that all air bubbles have been removed from syringes prior to intravenous injections. Introducing air into the vein can cause lethal lung, heart, or brain failure.

¹⁰Upon isolation of the spleens, it is important that the spleens are kept separate throughout the entire analysis. The total cells can be determined on a per mouse basis, number of T_{conv} thus increasing statistical power.

¹¹Alternatively, an anti-Fc receptor antibody (e.g., clone 2.4G2) can be used to block Fc receptors.

preventative model in which T_{regs} and T_{conv} same time. IBD is mediated by $CD4^+$ Th1 and Th17 cells (14,17), and thus this model assesses the capacity of the test T_{reg} population to control these T cell populations.

3.4.1. Induction of Colitis

1. Determine the number of $Rag1^{-/-}$ mice needed for the experiment (see Note 12).
2. On the day of the injection (Day 0) weigh the $Rag1^{-/-}$ mice using a digital scale (see Note 13).
3. Purify T_{conv} cells ($CD4^+$ $CD45RB^{\text{high}}$ $CD25^{-}$) cells from C57BL/6 mice by FACS as described in Subheading 3.1.
4. Following the sort, centrifuge cells at $300 \times g$ for 10 min and resuspend the T_{conv} in 2 ml of PBS + 2% FBS.
5. Count the cells using a hemocytometer and trypan blue staining to exclude the dead cells. Resuspend the T_{conv} cells in PBS + 2% FBS at 1×10^6 cells/ml.
6. Load the cells into a 3-ml syringe and inject $Rag1^{-/-}$ mice i.v. through tail vein with 5×10^5 T_{conv} cells (0.5 ml/mouse) using a 27-G needle (see Note 9).

3.4.2. Monitoring Body Weight

1. Weigh mice on the day of injection of T_{conv} cells and then once a week for 2–3 weeks. Once the mice start losing weight (over 2% body weight loss), monitor the mice daily for a sudden weight loss of up to 5% body weight, which is usually within a couple of days of the initial weight loss (see Note 13). In addition to weighing the mice, it is important to screen for clinical symptoms. Typical symptoms include lethargy, dehydration, hunched appearance, and diarrhea.
2. Percent weight change is calculated by comparing the current weight to the initial weight at day 0 as follows: percent weight change = $((\text{weight at day 0} - \text{current weight}) / \text{weight at day 0}) \times 100 \times -1.0$. For example, if the starting weight of the mouse at day 0 was 20 g and the current weight is 19 g, then percent weight change is calculated as follows: percent weight change = $((20 - 19) / 20) \times 100 \times -1.0 = -5\%$. This indicates that the mouse has lost 5% of its body weight. Typically, the mice start losing weight around 3–4 weeks post T_{conv} transfer.
3. When the mice have lost 5% of their body weight, prepare T_{regs} for transfer (see Notes 14 and ¹⁵). Purify T_{regs} ($CD4^+$ $CD45RB^{\text{low}}$ $CD25^+$) as described in

¹²The incidence of colitis upon naive T cell transfer can vary substantially between institutions (<20–100%) due to variations in the intestinal microbiota. Investigators should first perform a pilot experiment to determine incidence in their facility empirically. Typically, the greatest variable is *Helicobacter spp* colonization, but other strains are likely to influence colitis incidence (16,30,31). It is recommended to have at least five mice per experimental group. In *Helicobacter*-free mouse facilities or when using specific pathogen free mice, the incidence of colitis can be approximately 40%. In *Helicobacter*-positive mice, the incidence of the disease is greater. Therefore, assuming that there will be at least a 50% incidence of colitis following T_{conv} injection, and there are three groups of T_{regs} to be assessed for their function (e.g., No T_{reg} , wild type T_{reg} , and experimental T_{reg}), use at least 30 $Rag1^{-/-}$ mice per experiment. $Rag1^{-/-}$ mice are the most common strain used for these experiments but in theory any lymphopenic strain could be used. However, this would need to be verified empirically.

¹³It is important that the weight of the mice is measured as accurately as possible. Body weight of the mice should be taken every week on the same day and preferably at the same time using the same weighing balance during the entire course of the study to avoid any artifacts. Use a reference of known weight (such as a plastic lid or a tube filled with a set volume of water) to ensure the balance is accurate each time before and after the mice are weighed.

¹⁴The time window for optimal T_{reg} adoptive transfer is small (1–2 days) so careful planning is critical. The availability and flexibility of your institutional sorting facilities should be considered in this planning process. Planning should be done in advance as much as possible. Typically, the mice will not reach 5% weight loss at the same time so several sorts over consecutive days are often required (~3–4 days, 3–4 weeks from T_{conv} cells injection). Do not set up different groups of T_{regs} on different days but rather a few from each group of T_{regs} on each day.

Subheading 3.1. Count the cells using a hemocytometer and trypan blue staining to exclude dead cells. Centrifuge cells at $300 \times g$ for 10 min and resuspend the T_{regs} cells in PBS + 2% FBS at 1.5×10^6 cells/ml.

4. Load the cells into a 3-ml syringe and inject *Rag1*^{-/-} mice intraperitoneally (i.p.) with 7.5×10^5 T_{regs} (0.5 ml/mouse) using a 27-G needle.
5. Tabulate the body weight of mice at the time of T_{reg} injection. Separate mice into experimental groups (i.e., wild type T_{reg} , experimental T_{reg} or no T_{reg} group) with similar percent weight loss among groups prior to T_{reg} injection.
6. The body weight of the mouse at the point of T_{reg} injection is taken as the starting weight for further assessment of disease progression or recovery. Thus, the percent weight change following T_{reg} injection is calculated as follows: percent weight change = ((weight at the time of injection of T_{regs} - current weight)/weight at the time of injection of T_{regs}) $\times 100 \times -1.0$. Accurate monitoring of body weight provides an indication of whether the mouse has recovered from colitis or not. Weigh mice every 7 days from the day of T_{reg} injection for 4 weeks and tabulate the weights (see Note 13).

3.4.3. Analysis of Experimental Mice

1. Four weeks following injection of T_{regs} , the mice are euthanized, and the spleen and mesenteric lymph nodes are collected into separate wells of a 24-well plate containing 1 ml HBSS for flow cytometric analysis.
2. Tease apart each spleen and mesenteric lymph nodes separately as described in Subheading 3.1 and stain 200 μ l of cells first with 10% normal mouse sera in FACS buffer for 5 min on ice to block the Fc receptors and then stain for CD4, CD25, CD44, CD62L (to distinguish memory and naïve T cells), CD69 (early activation marker), and Foxp3 (to detect T_{regs}) in a 96-well V-bottom plate for 20 min on ice.
3. Centrifuge cells at $300 \times g$ for 2 min and wash twice with FACS buffer.
4. Resuspend cells in 100 μ l of FACS buffer and analyze by flow cytometry to determine the percentages of T_{regs} and T_{conv} .

3.4.4. Preparing the Colon for Histological Analysis—The colon can be prepared for histological analysis at the same time the spleen and mesenteric lymph nodes are collected.

1. Cut the colon from just above the rectum using scissors.
2. Using forceps gently tweeze out the colon so that it separates from the attached connective tissues.
3. Cut again just below the cecum to obtain the colon which is now untangled from the connective tissue. It is important that this procedure is carried out as consistently as possible among the individual mice as the severity of the disease shortens the length of the colon, which can be measured and tabulated.
4. Hold the colon straight at one end using a forceps. Use a 10-ml syringe filled with 10% neutral buffered formalin <!-- Caution: Irritant and suspected carcinogen. Perform with caution when flushing out the fecal matter as the formalin can spray over the personnel performing this procedure. Use eye protection or perform this

¹⁵It is important to inject the mice as soon as they lose 5% of their body weight to minimize differences in the severity of disease between the mice used for T_{reg} injection

step in a fume hood. Dispose of the formalin waste as per your institutional guidelines. Refer to the manufacturer's MSDS for more details.) attached to a 23-G needle to flush out the fecal matter through the length of the colon into an empty waste container.

5. Once the colon is clear of fecal matter, the tissue is placed in a numbered tissue cassette and stored in formalin until all the different groups are collected 4 weeks post T_{reg} injection. Samples should be paraffin-embedded, sectioned at 5 μ m, and stained with Haematoxylin and Eosin (H&E) following standard histological protocols (see Note 16).

3.4.5. Microscopic Analysis and Scoring of the Colonic Tissue—It is important that the severity of the inflammation is assessed and scored in a blinded manner. Typically the score ranges between 0 and 5, where a score of 0 is given when there is no inflammation and a score of 5 denotes severe ulceration, diffuse transmural inflammation, and crypt loss. Details of the different scores are as follows:

Score 0	No Inflammation.
Score 1	Minimal inflammation, multifocal infiltrates in the lamina propria.
Score 2	Mild inflammation in the lamina propria and submucosa.
Score 3	Moderate inflammation in the lamina propria, sub mucosa, focally transmural, mucosal hyperplasia, minimal necrosis, focal ulcers, and mucin depletion.
Score 4	Severe focally extensive inflammation, transmural, crypt necrosis/loss, epithelial hyperplasia, erosions, some ulcers, mucin depletion.
Score 5	Ulceration, loss of crypts, severe diffuse transmural inflammation.

3.4.6. Representing Weight Loss and Histological Scores

1. Weight loss is usually graphed using the mean of the weights plus the standard error of the mean from the different groups (i.e., wild type T_{reg} , no T_{reg} or experimental T_{reg} group). For the purpose of monitoring, the recovery of mice from weight loss, the starting weight is taken as the weight at which the mice are given T_{regs} . Mice given wild type T_{regs} will start recovering with In contrast, T_{regs} defective in their function evident weight gain. will not be able to alleviate weight loss and the mucosal inflammation. The control group, which did not receive T_{reg} (no T_{reg} group), will also continue to lose weight (see Note 17).
2. Histological score (mean and standard error of mean) between 0 and 5 is plotted for each group.

3.5. Experimental Autoimmune Encephalomyelitis (EAE) Model

EAE is a useful and well developed murine model of the human autoimmune disease, multiple sclerosis. Since T_{regs} can contribute significantly to the reduction and control of the disease in mice (18,19), EAE is a valuable system to assess the function of in T_{regs} vivo. Although the protocol requires daily disease monitoring, the data obtained can potentially reveal small differences in T_{reg} efficacy either through disease score, disease incidence, or

¹⁶Typically, tissue sections are stained with H&E to determine cellular infiltration and tissue destruction. However, Alcian blue/ PAS staining (to distinguish between acidic and neutral mucin in the colonic tissue and aid in goblet cell identification), anti-CD3, and Foxp3 staining (to detect T_{conv} and T_{regs}) may also be used on the tissue sections.

¹⁷Most IACUC protocols require the euthanasia of mice that lose over 10% of their body weight.

disease kinetics. EAE can be induced with several peptide and protein antigens derived from the CNS of mice. However, this protocol is limited to the description of MOG35–55 immunization of C57BL/6 mice, as it allows for the use of multiple genetically modified mouse strains available on the C57BL/6 background. EAE is mediated by CD4⁺ Th1 and Th17 cells (20–22), and thus this model assesses the capacity of the test T_{reg} population to control these T cell populations.

3.5.1. Injection of T_{regs}

1. T_{regs} are injected the day before EAE disease induction. Separate the mice into experimental groups (i.e., mice that will not receive T_{regs}, mice that will receive control T_{reg}, and mice that will receive experimental T_{regs}), and mark each mouse either by ear tag or ear clipping. Normally, five mice per experimental group are used in an experiment.
2. Sort T_{regs} and T_{conv} cells as described in Subheading 3.1. It is advisable to use mice with different congenic markers such as B6.PL mice (mice that express the congenic marker Thy1.1) or B6.SJL-*Ptpca Pep3b*/BoyJ mice (mice that express the congenic marker, CD45.1), if brain infiltrating T cells will be analyzed.
3. Following the sort, centrifuge cells at 300 × g for 10 min and resuspend the T_{regs} in 1 ml of PBS + 2% FBS.
4. Count the cells using a hemocytometer and trypan blue to exclude dead cells.
5. Dilute the T_{regs} to 5 × 10⁶ cells/ml with PBS + 0.1% FBS.
6. Inject 200 µl T_{regs} (1 × 10⁶) i.v. (see Notes 9 and 18).

3.5.2. Preparing the CFA/MOG35–55 Peptide Emulsion

1. Prepare 4 mg/ml Complete Freund's Adjuvant (CFA) <!--> Caution: CFA is an inflammatory reagent. Avoid skin or eye exposure. Self injection can cause a positive PPT test and lead to a granulomatus reaction and skin lesion. Use gloves and protective eyewear while handling CFA. Refer to the manufacturer's MSDS for more details.) by diluting 100 mg of heat killed *Mycobacterium tuberculosis* in 25 ml of IFA. Mix the solution using a frosted glass tissue homogenizer. The solution can be stored at 4°C for at least 1 month. Prior to each use, mix CFA thoroughly as the bacterium tends to settle to the bottom of the vial.
2. The emulsion is made the day before the injections. Make the emulsion at 1:1 ratio of CFA to peptide diluted in PBS. The final concentration of *M. tuberculosis* in the emulsion will be 2 mg/ml. To make the emulsion, load the appropriate amount of CFA (0.5–1 ml) into one 2-ml glass syringe (the volume of CFA should not exceed one half of the syringe), expunge the air and lock with connector, set aside.
3. Load an equal amount by volume of the peptide into another 2 ml glass syringe, expunge the air and connect to the other syringe from step 2 through the connector.
4. Carefully mix the two solutions. Always start by completely pushing the peptide into the CFA. Then continue pushing the mixture back and forth between the two syringes for at least 10 min. Cool the emulsion at –20°C for 5 min, mix again for 10 min, and leave at 4°C overnight.

¹⁸Significant decrease in disease symptoms has been observed after injection of 1 × 10⁶ natural T_{regs} (19). Higher (up to 4 × 10⁶) or lower (0.5 × 10⁶) numbers of T_{regs} can also be used.

5. The next day remix the emulsion prior to injections. The sudden increase in resistance in the syringe during the mixing indicates that an emulsion has formed (see Note 19).
6. Expunge the emulsion completely into one of the syringes, exchange the empty syringe with a 1-ml syringe, and carefully load the emulsion into the 1 ml syringe for injections.
7. Attach the 25G needle to 1 ml syringe containing the emulsion and force out any air bubbles.

3.5.3. Immunization of Mice for EAE Induction

1. Prepare 1 ml syringes loaded with peptide emulsion for subcutaneous (s.c.) injections and pertussis toxin for i.p. injections. For example, for injection of 15 mice, prepare five syringes each loaded with 600 μ l of emulsion and five syringes loaded with 600 μ l of pertussis toxin.
2. To inject the mice, anesthetize mice in an isoflurane chamber or have a second person hold the mouse by the nap of the neck and at the base of the tail and gently stretch the mouse over the cage bar lid, taking care not to injure or suffocate the mouse.
3. Inject 50 μ l emulsion s.c. into both shoulder pads and both flanks (a total of 200 μ l containing 100 μ g of peptide and 400 μ g of CFA).
4. Inject 200 μ l of 1 μ g/ml *Bordetella pertussis* toxin diluted in PBS i.p..
5. After 48 h administer another 200 μ l of pertussis toxin i.p..

3.5.4. Monitoring Disease—Monitor mice daily starting at day 8 post immunization (see Note 20). Assign clinical scores based on the following criteria (see Note 21):

- Score 0** No obvious physical motor differences are observed when compared with the unimmunized mouse. When the mouse is picked up, the tail has tension and the feet are separated.
- Score 1** Complete flaccidity of the tail or hind limb weakness (not both). A weak tail and an unsteady gait are the initial signs of paralysis. When the mouse is placed on top of the cage bar lid, the tail will fall between the bars or hang flaccidly over the edge of the cage. To verify complete flaccidity, the tail can be flicked in the upward direction. In a healthy mouse, the tail will stay partially erect and will not immediately fall down. Additionally, when the mouse is picked up by the tail, a paralyzed mouse will hang straight, with no tail rigidity or curving of the tail base. The hind limb weakness usually presents as an unsteady walk and slipping of the mouse's hind limbs between the bars of the cage lid. Hind limb weakness can be present in the absence of the flaccid tail and should be scored as 1 or 1.5, if there is partial tail paralysis.

¹⁹The length of emulsion formation varies significantly and might be affected by the salt concentration and purity of the peptide. Cooling the syringes filled with CFA and peptide to 4°C helps in emulsion formation. The quality of the emulsion can be tested by dropping a small amount on the surface of a beaker of water. If the emulsion is good, it will remain intact on the water surface. If not, the oil component will rapidly spread out over the entire water surface and the drop will disintegrate.

²⁰Mice usually develop symptoms between days 10 and 18, and the experiment can be terminated 30–40 days post immunization.

²¹Since the scoring system is subjective, the scoring should be performed in a blinded manner (i.e., the identity of the experimental groups should be hidden from the person who is scoring the mice). Additionally, the same person should score the mice throughout the experiment to prevent person to person variability in scoring.

- Score 2** Both limp tail and hind limb weakness or partial paralysis. In addition to monitoring hind limb weakness, another early sign of paralysis is the loss of the righting reflex. When a healthy mouse is put on its back, it quickly flips to the upright position. A sick mouse may have slow to complete impairment in the righting reflex. In the absence of other signs, impairment of the righting reflex of any grade is scored as 2.
- Score 3** Total hind limb paralysis. The mouse can no longer use hind limbs to maintain rump posture or walk. The mouse is able to move hind legs to some degree, but if put on top of the cage bar lid, the feet will fall through and it will be unable to pick them back up.
- Score 4** Hind limb paralysis and front limb weakness/paralysis. With the total loss of movement in hind limbs, the mouse drags itself only on its forelimbs. Mice appear alert and feeding, but do not move around the cage. Mice at this stage should be given food on the cage floor, water bottles with long sipper tubes, and daily subcutaneous saline injections to prevent death by dehydration.
- Score 5** Moribund. Mice at this stage are not feeding, not alert, and close to death. If the mouse is scored 5, it should be immediately euthanized. After a mouse is given a score of 5, the same score is entered for the rest of the duration of the experiment (see Note 22).

Half scores can be given, if the clinical symptoms fall in between the two scores (i.e., if the symptoms appear to affect only one side of the mouse). Expect the experimental group to have scores ranging between 2 and 3 at the peak of disease. The normal or wildtype T_{reg} treated group should have scores between 1 and 2 (see Note 23).

3.5.5. Data Analysis—Data can be graphed as the average of the clinical scores of all mice in one experimental group (y-axis) against the day post immunization (x-axis). Additionally, incidence can be graphed as percent of mice presenting any clinical symptoms (y-axis) vs. days post immunization (x-axis).

3.5.6. Analysis of Brain-Infiltrating Lymphocytes—The brain and spinal cord are both targets of cellular infiltration. A significantly larger number of cells can be obtained from the brain than the spinal cord with limited technical difficulty when compared with spinal cord dissection. If one wishes to analyze the phenotype or perform functional analyses with the lymphocytes infiltrating the brain, the following protocol can be performed.

1. Sacrifice mice by CO₂ inhalation or a similar method as approved by IACUC guidelines.
2. Place the mouse on its stomach and spray with 70% ethanol.
3. Using surgical scissors, make a small incision through the skin on the back below the neck area and remove the skin revealing the scalp.

²²Most institutions require euthanasia of a mouse after several days at a score of 4. At the point of euthanasia, the mouse is scored as 5. Contact your IACUC committee for protocol approval and institutional EAE guidelines.

²³Usually, injection of 100 µg of MOG35–55 results in a peak average score of 2.5. The peptide activity can vary between batches, so an optimal concentration of peptide should be determined based on disease symptoms. Ideally, an HPLC grade purified peptide should be used. If you have difficulty inducing EAE, check that the mice are handled gently, and that they are not housed under stressful conditions. Additionally, the mice should be rested for at least 7 days after arrival to your animal facility. If necessary, increase the amount of peptide antigen.

4. Gently cut the skull bone around the perimeter of the scalp starting at the back base of the skull and moving forward toward the front of the head. Flip the top part of the skull from the back toward the front of the head and expose the brain.
5. Remove the brain and transfer into a conical tube containing PBS or HBSS.
6. Create a single cell suspension of the brain tissue by homogenizing it through a 40- μ M cell strainer into a 50-ml conical tube with the plunger of a 1-ml syringe.
7. Centrifuge homogenate at $300 \times g$ for 10 min at 4°C.
8. Resuspend homogenate in 7 ml of room temperature HBSS.
9. Dilute 100% Percoll to 90% and 70% by volume in PBS.
10. Add 3 ml of 90% Percoll to tubes containing 7 ml of homogenate and invert to mix and make a 27% Percoll solution.
11. Carefully underlay with 70% Percoll.
12. Centrifuge at $415 \times g$ (2,500 rpm) for 25 min at 18°C without brakes.
13. Transfer the cells at the 27/70% interface to a new 15 ml tube.
14. Fill the tube with culture media and centrifuge at $300 \times g$ for 10 min at 4°C.
15. At this point the brain cellular infiltrate is ready for analysis by flow cytometry or in vitro assays.

3.6. B16 Melanoma Model

B16 cells are weakly immunogenic owing to their reduced MHC I expression (23). The parent B16 line (B16–100K) is nonmetastatic and develops a well encapsulated intradermal (i.d.) tumor. Metastatic variants of the dermal parent line including lung and liver metastatic cell lines have been developed to study eradication of metastatic tumors (24,25). The B16F10 mouse melanoma cell line was originally provided by Isaiah Fidler (MD Anderson Cancer Center, Houston, TX) and passaged intradermally in mice four times at a dose of either 100,000 cells (referred to as B16–100K) or 25,000 cells (referred to as B16–25K) (26) to ensure reproducible and aggressive i.d. tumor growth at the specified cell dose. The B16–25K cell was found to grow more reproducibly as lung metastases, so this line was chosen for future experiments involving intravenous tumor cell inoculation. Previous studies shown that T_{regs} prevent anti-tumor immunity against the have poorly immunogenic B16 melanoma (26,27). Wild type naïve $CD4^+CD25^-$ and $CD8^+$ T cells alone or in combination with T_{regs} are adoptively transferred into *Rag1*^{-/-} mice. The following day, mice are challenged with an i.d. inoculation of B16–100K cells. Tumor size is monitored daily to determine the effect of the T_{regs} on tumor burden. Because of variations in tumor size, we suggest having at least five mice per group. Concomitant immunity can be further assessed by surgical excision of the primary tumor, followed by secondary challenge with B16 i.d. at a remote site, or a metastatic variant of B16, B16–25K, i.v. to assess lung metastases. Tumor clearance in the B16 model is mediated by $CD8^+$ T cells (26,27), and thus this model assesses the capacity of the test T_{reg} population to suppress $CD8^+$ T cells.

3.6.1. Culture of B16 Melanoma Cells—B16 cells should be thawed 4–5 days prior to tumor challenge and maintained at a low passage to ensure good viability. B16 cells are an adherent cell line and should adhere to culture flasks within 1 day. Cells that are slow to adhere or do not adhere should not be cultured or used in assays. As cell density is critical to the proper growth and viability of B16 cells, it is important to seed cells at multiple concentrations to ensure that at least one flask will be optimal for inoculation.

1. Add 26, 27, 28, and 29 ml of B16 culture media to each of four T175 flasks (see Notes 24 and ²⁵).
2. Remove 1 vial of B16 cells containing approximately $4\text{--}5 \times 10^6$ cells in 1 ml from liquid N₂.
3. Thaw cells by holding and shaking in a 37°C water bath for approximately 30 s.
4. As soon as the freeze media thaws, transfer the contents of 1 vial into a 50-ml conical tube containing 19 ml of B16 culture media.
5. Invert tube to mix.
6. Transfer 1, 2, 3, or 4 ml of cells into each of the four T175 flasks for a total volume of 30 ml per flask.
7. Shake flasks to mix, making sure the medium completely covers the bottom of each flask.
8. Culture cells in an incubator at 37°C, 5% CO₂ for 4–5 days. One day before tumor challenge, aspirate media from flasks and replenish flasks with fresh, B16 culture media (prewarmed in a 37°C water bath).

3.6.2. Adoptive Transfer of T cells—*RagI*^{−/−} mice are reconstituted with 9×10^6 CD4⁺CD25[−] T cells, 6×10^6 CD8⁺ T cells, and 1×10^6 T_{regs} (in desired groups).

1. Purify CD4⁺CD25[−] T cells, CD8⁺ T cells, and T_{regs} from desired source as described in Subheading 3.1 (see Note 26).
2. Count all cells and adjust in murine T cell culture medium (see materials Subheading 2.1) to 9×10^6 /ml (CD4⁺CD25[−] T cells), 6×10^6 /ml (CD8⁺ T cells), and 1×10^6 /ml (T_{regs}).
3. For each group, combine 1 ml of cells per mouse into a 15-ml conical tube. For example, an experiment that includes five mice per group with two groups (Group A: with T_{regs} Group B: and without T_{regs}) will be divided into two tubes. Each tube will contain 5 ml of CD4⁺CD25[−] T cells and 5 ml of CD8⁺ T cells. In addition, add 5 ml of T_{regs} to the tube containing Group A cells.
4. Centrifuge cells at $300 \times g$ for 10 min and aspirate supernatant.
5. Resuspend cells in sterile PBS + 2% FBS at 0.3 ml per mouse to be injected. Include an additional 10% of volume PBS + 2% FBS to account for minor losses that occur when loading the syringes for injections. For example, Groups A and B will each be resuspended in 1.65 ml PBS + 2% FBS. ($0.3 \text{ ml/mouse} \times 5 \text{ mice}$) + 0.15 ml = 1.65 ml
6. Attach a blunt needle to a sterile 3 ml disposable syringe and pull cells into syringe by drawing up plunger.
7. Remove blunt needle and replace with sterile 27G needle. Maintain sterility of cells at all times.

²⁴B16 cells should be frozen at $4\text{--}5 \times 10^6$ /ml in 1 ml aliquots of 10% DMSO in FBS. The B16 thawing protocol is based upon a highly concentrated B16 cell frozen stock ($4\text{--}5 \times 10^6$ /ml/ vial) and should be used only as a guide. The proper dilution volume must be determined empirically, based upon the density of the frozen stock and the growth characteristics of the cells postthaw. B16 cells takes approximately 18 h to double in culture.

²⁵One T175 flask at 75–80% confluence will provide enough cells to challenge approximately 25 mice.

²⁶It is not necessary to include the CD45RB antibody in the purification of CD4⁺ T cells for B16 tumor experiments. Instead, splenocytes should be stained with anti-CD8 antibody in addition to CD4 and CD25 to facilitate purification of CD8⁺ T cells.

8. Inject cells i.v. into the tail vein of the mice (see Note 9).

3.6.3. B16 Melanoma Cell Preparation—One day following adoptive transfer of T cells into *Rag1*^{-/-} mice, challenge the mice with the B16 melanoma (see Note 27). Each mouse will receive 1.2×10^5 B16 cells intradermally in the rear flank.

1. Place sterile PBS and frozen Trypsin-EDTA aliquots (7 ml per T175 flask) in 37°C water bath for 15–20 min to thaw and warm.
2. Place flasks of B16 cells under microscope to determine health and confluency of cells. To ensure good viability, cells should be harvested when flasks reach no more than 75–85% confluence.
3. Determine the best dilution(s) of cells for harvest. Cells should be about 70% confluent and be well adhered to the flask. A flask that contains cells that are clumpy or have died and are floating should not be used.
4. Aspirate media from flasks and wash cell monolayer with 15 ml warm PBS. Repeat.
5. Add 7 ml of Trypsin-EDTA per flask and swirl to coat the cells.
6. After about 30 s, forcefully tap flasks to release cells from flask. To confirm that the cells have been released, visualize cells under the microscope.
7. Immediately add 12 ml cold B16 culture media to quench the Trypsin-EDTA. It is important to follow this trypsinization and quenching protocol exactly as over trypsinizing cells will decrease cell viability.
8. Transfer cells to 50 ml conical tube(s) and centrifuge at $300 \times g$ for 5 min at 4°C.
9. Wash cells twice in cold RPMI without any additives.
10. Before final spin, count cells by trypan blue exclusion using a hemocytometer. Viability should be >95%. If viability is <95%, the cells should not be used.
11. Resuspend cells at 2.4×10^6 /ml in cold RPMI without any additives. This equates to 1.2×10^5 B16 cells per 50 μ l.
12. Aliquot cells into 2 ml cryo vials or 1.5 ml Eppendorf tubes for easy loading of syringes and to avoid over-mixing of cells during inoculation.
13. Place cells on ice and maintain on ice for duration of the inoculation.

3.6.4. B16 Inoculation and Measurement Primary Tumor Growth—Because of the technical difficulty of the tumor inoculation, mice must be anesthetized with isoflurane for the procedure (see *of* Note 28). In theory, alternate anesthetics can be used if so desired; however, their use must be determined empirically. The presence of fur hinders proper measurement of the tumor size; therefore, fur must be removed from the site of tumor injection, using a small electric razor. The use of Nair™ or other depilatory can cause a minor inflammatory reaction on the skin and is therefore not recommended.

1. Mix cells by vortexing briefly. Draw cells into 1 ml syringe fitted with 30G needle. Remove air bubbles and set syringe aside.

²⁷It is important to note that most IACUC guidelines require that all cell lines must be tested for murine virus contaminants prior to injecting into mice.

²⁸Isoflurane (1-chloro-2, 2,2-trifluoroethyl difluoromethyl) is a general inhalation anesthetic drug. Institutional approval is required for use and proper training should be completed prior to use. Although isoflurane has the largest margin of safety of all potent halogenated agents, care should be taken to avoid excessive exposure.

2. Anesthetize mouse with isoflurane using a chamber design stationary anesthesia machine. When a mouse is sufficiently anesthetized, its respiratory rate decreases and has no reaction to a pinch of the toe.
3. Transfer anesthetized mouse from chamber to a clean work space nearby. Place the mouse on its left side. Using a small electric razor, shave the fur from the hind leg up to just below the front leg (approximately 12×12 mm patch). See photograph in Fig. 2a.
4. Inject 1.2×10^5 B16 cells in 50 μ l of RPMI i.d. on shaved right flank Fig. 2a. It is important that cells are injected i.d. and not subcutaneously. Subcutaneous tumors cannot be surgically excised; therefore, no secondary challenge (or analysis of tumor infiltrating lymphocytes) is possible if inoculated subcutaneously. If i.d. inoculations are done properly, a small, liquid-filled bubble will appear on the skin, and the bubble will lift up with the skin when the skin is pulled away from the mouse.
5. Transfer mouse into a clean cage that was prewarmed with a heating pad and monitor recovery. Mouse should be mobile and active within a minute or so following tumor inoculation (see Note 29).
6. Tumors will develop 5–7 days post challenge. At the first sight of a tumor, all tumors must be measured daily using a dial caliper.
7. Tumor size can be reported in diameter (mm) or in total area (mm^3). Measure length and width of tumor in mm to report diameter or calculate total area of tumor using the following equation: $(a^2 \times b)/2$, where a is the smaller caliper measurement and b the larger).
8. Tumors should be excised when a tumor ulcerates, reaches a maximal diameter of 10 mm (500 mm^3), when discomfort or impaired mobility is noted or as set by IACUC guidelines.

3.6.5. Surgical Excision of Tumor and Secondary Challenge—Intradermal primary tumors should be surgically excised at 5–10 mm diameter. Less than 5% of primary tumors should recur following surgery. Any mice with recurrent primary tumors should be omitted from the concomitant tumor study (27). As with the primary tumor challenge, anesthetize mouse with isoflurane and monitor breathing rate and reflexes throughout the procedure. To maintain sterility, surgery is performed in a laminar flow hood. Mice must remain anesthetized during surgery; therefore, a mobile isoflurane machine attached to a breathing tube must be set up within the hood.

1. Anesthetize mice with isoflurane. When mice are sufficiently anesthetized, transfer mice (1 mouse at a time) from the isoflurane chamber into a laminar flow hood.
2. Place the mouse on its back with its nose securely fitted into the breathing tube.
3. Place gauze or a paper towel under mouse to prevent movement on the slick surface of the hood during surgery.
4. Swab tumor and surrounding skin with a Q-tip coated in surgical iodine solution. Beginning in the center of the tumor and working toward the edges, swab the tumor in a circular motion.

²⁹Following isoflurane anesthesia, animals have reduced body temperature, which can cause distress. To aid in recovery, mouse cages can be placed on a heating pad or under a heat lamp. Institutional guidelines may require additional monitoring, so it is imperative to check with your institution for instructions.

5. Swab area with a single use alcohol pad.
6. Repeat iodine and alcohol cleaning of the area.
7. Loosely grasp the tumor with blunt forceps.
8. Using surgical scissors make a small incision through the skin near the tumor and gently cut around the tumor to remove the tumor together with a 2-mm perimeter of healthy skin. This margin will ensure that the tumor does not recur.
9. The tumor can be kept for analysis of infiltrating lymphocytes as detailed in Subheading 3.6.6.
10. Join remaining skin with forceps and close the wound with 2–3 steel wound clips.
11. Liberally apply Neosporin triple antibiotic ointment (or any triple antibiotic ointment) to wound using a fresh Q-tip.
12. To help control pain, inject 0.1 mg (0.1 ml of 0.015 mg/ml) of buprenorphine or 0.15 mg (0.05 ml of 2.5 mg/ml) of rimadyl subcutaneously in the upper dorsal skin between the shoulders in the back of the head.
13. Transfer mouse to a clean cage that has been prewarmed with a heating pad or is under a heat lamp.
14. Alternatively, if a tumor challenge is desired at a remote site in the same animal, transfer the mouse to a sterile work space, and shave the fur off of the mouse on opposite side of primary tumor.
15. Administer secondary i.d. tumor challenge as described above for primary tumor (Subheading 3.4.4). Alternatively, the B16–25K metastatic cell line can be injected i.v. (1.2×10^5 cells in 100 μ l PBS) to initiate a metastasis study.
16. Monitor mice for recovery from anesthesia. Recovery from the prolonged anesthesia that is required for surgical excision is slower than that following the primary challenge. Mice should be mobile and active within 2–3 min following cessation of anesthesia (see Note 29).
17. After 5–7 days, surgical wounds will be healed and wound clips can be removed. Remove clips using a wound clip remover.

3.6.6. Analysis of Dermal Tumor-Infiltrating Lymphocytes—If one wishes to analyze the phenotype or perform functional analyses with the tumor infiltrating lymphocytes, the following protocol can be performed. Tumors can be surgically excised as detailed in Subheading 3.6.5 or can be analyzed as an endpoint following sacrifice of the animal.

1. Excise tumor as described earlier and transfer into a sterile 24-well tissue culture plate containing PBS.
2. Create single cell suspension of tumor tissue by teasing tumor between frosted microscope slides. Wash plate and slides with 1 ml PBS and transfer into a 15-ml tube.
3. Centrifuge homogenate at $300 \times g$ for 10 min at 4°C.
4. Resuspend homogenate in 2 ml of room temperature 80% Percoll [100% Percoll: 90 ml neat Percoll + 10 ml 10 $\times$ HBSS (both sterile). Dilute 100% Percoll in 1 $\times$ HBSS to create 80% and 40% solutions].
5. Transfer cells in 80% Percoll to a new 15-ml tube.

6. *SLOWLY* layer 2 ml of 40% Percoll on top of the 80% solution. Be careful not to disturb the interface.
7. Centrifuge at $415 \times g$ for 25 min at 18°C without brakes.
8. Transfer the cells on the 40/80% interface to a new 15-ml tube using a glass pipet or a longer pipet tip.
9. Fill the tube with B16 culture media and centrifuge at $300 \times g$ for 10 min at 4°C.
10. Resuspend in PBS + 2% FBS containing antibodies of interest and incubate for 20 min on ice.
11. Centrifuge cells at $300 \times g$ for 10 min at 4°C and proceed with purification by FACS.

3.6.7. Analysis of Lung Metastases—Lung metastases will develop 22–28 days post inoculation. Since metastases are not outwardly visible, a sentinel mouse must be sacrificed to determine presence of metastases. We therefore recommend inoculating 2–3 extra mice (in addition to the experimental mice) that can be used to assess the presence of metastases.

1. Prepare 10 ml of 5% H₂O₂ dish and two, 5 cm tissue culture in PBS in a 5-cm tissue culture dishes with 10 ml PBS only.
2. Sacrifice mice by CO₂ inhalation or a similar method as established by IACUC guidelines.
3. Place the mouse on its back and spray with 70% ethanol. Cut mouse open to reveal the lungs in the thoracic cavity.
4. Open the rib cage, revealing the lungs. Remove the lungs and place directly in a dish containing PBS for 1 min, transfer to in PBS for 1 min, and then transfer dish containing 5% H₂O₂ to dish containing fresh PBS for 1 min. This process will inflate the lungs and render metastases more visible.
5. Dissect the lungs into lobes and count both sides for black pigmented spots, which are metastases. Metastases are readily visible by eye. If desired, a dissection microscope can be used to aid in counting.
6. If more than 40 metastases are visible, the lungs can also be weighed as a quantitative measurement.

3.7. Foxp3[−] Rescue Model

Mice that lack Foxp3 develop a rapid, multiorgan lymphoproliferative disease that results in lethality 16–25 days after birth (28). Transfer of 1×10^6 natural T_{regs} into 1–3 day old Foxp3[−] pups can protect them from the lethal autoimmune disease for at least 5 weeks (29). However, the use of more than 1×10^6 T_{regs} may provide longer, more significant protection. Optimal cell number must be determined by the investigator. Because of the limited window of opportunity for this transfer (T_{regs} injected beyond 3 days of life will not prevent disease), timed breeding, rapid genotyping of litters, and open access to cell sorting facilities to isolate T_{regs} are critical components of a successful experiment. Although initiating a large experiment is challenging, we suggest having at least 5 mice per group. This model assesses the capacity of the test T_{reg} population to control CD4⁺ and CD8⁺ T cell lymphoproliferation and autoimmune destruction that underlies the disease manifested in Foxp3[−] mice (29).

3.7.1. Breeding and Genotyping Considerations—Male Foxp3[−] mice do not survive long enough to breed; therefore, Foxp3^{+/-} (heterozygous) female mice crossed to male

C57BL/6 mice must be used as breeders. Genotypic distribution of the mutant allele does not follow expected Mendelian genetics. Only approximately 10–20% of the offspring are male knockouts; therefore, a large number of breeder pairs may be needed to generate the number of mice needed for even a small experiment. For example, from 5 breeder pairs, one might expect 5–10 pups per month. This must be taken into consideration when planning experiments.

Careful monitoring and timing of breeding is critical for the success of this experiment. Our experience suggests that litters are born approximately 19 days after a plug is visible in *Foxp3*^{+/-} mice; however, this can vary from institution to institution, so gestational length must be determined by the investigator. On the day that they are born, litters must be sexed and males must be tagged (typically 1 toe is removed as ear tagging at this age is not possible). Newborn male mice are distinguished by the presence of a small black dot at the base of the tail on the front side of the mouse (Fig. 2b). In addition, tails must be clipped, digested, DNA extracted, and genotyping performed within 24 h. These necessities must be considered and accounted for in the planning of these experiments as T_{regs} must be adoptively transferred into day 2 (ideally) or day 3 old mice.

3.7.2. Adoptive Transfer of T_{regs}

1. Isolate T_{regs} as described in Subheading 3.1
2. Count cells by trypan blue exclusion using a hemocytometer and resuspend 1×10^6 T_{regs} in 30 μ l per mouse sterile PBS + 2% FBS.
3. Because of the small volume to be injected (30 μ l per mouse), it is important to transfer cells into a tube/vial into which a small needle can be inserted to load the syringe for inoculations. The lid of a 2-ml cryo vial works very well for this purpose.
4. Pull cells from the lid of a 2-ml cryo vial into an insulin syringe fitted with a 30-G needle by drawing up plunger. Maintain sterility of cells at all times.
5. Cells should be injected i.p. into mice. Cells injected i.p. directly through the abdominal wall are prone to leaking out when the needle is removed. Instead, injecting into the peritoneal cavity through the front side of the hind leg, parallel to the quadriceps, works well (Fig. 2c and d).

3.7.3. Macroscopic Analysis of *Foxp3*⁻ Mice—By 10–14 days of life, untreated *Foxp3*⁻ mice are distinguishable from their wild-type littermate controls, based on their small stature, hunched posture, scruffy fur, and scaly erythematous skin on the ears and tail. The disease progresses such that 20–25 day old mice are terminally ill and moribund. Histological analysis of lungs, liver, and skin (ear) reveals severe inflammation and destruction of tissue architecture marked by inflammatory cell infiltrations in the liver, lungs, and skin dermis. At the cellular level, untreated *Foxp3*⁻ mice have splenomegaly and severe lymphadenopathy with a two to threefold increase in T cells compared with wild type littermates. To fully dissect the pathological manifestations of the disease and the ability of T_{regs} to prevent such disease, both macroscopic and microscopic analysis is needed. Typically, we perform this analysis at 3.5–4 weeks of age.

1. Macroscopic analysis of mice can be performed by inspection of the mouse's external appearance. A 6 point scoring system is used to describe a mouse's external appearance.
 - i. Is the mouse runted (small)? No: 0 point; Yes: 1 point
 - ii. Is the mouse's tail scaly and/or with lesions? No: 0 point; Yes: 1 point

- iii. Are the mouse's ears small in size, scaly, and/or with lesions?; No: 0 point; Yes: 1 point
 - iv. Are the mouse's eyelids scaly, not fully open?; No: 0 point; Yes: 1 point
 - v. What is the activity level of the mouse? Normal: 0 point; Moderately impaired mobility: 1 point; Immobile: 2 points
2. As the number of male *Foxp3*⁻ mice born per litter is small, it is often necessary to analyze mice on multiple days. Therefore, it is important to have a means to compare mice. This is accomplished by taking the following photographs next to a ruler or other scale bar: (a) whole mice from the relevant groups laying on their sides and (b) spleen and lymph nodes (inguinal, axillary and cervical) lined up next to each other from the relevant groups (Fig. 2e and f) (see Notes 30 and ³¹).
 3. Microscopic analysis involves both histological analysis and analysis of cellularity of the spleen and lymph nodes (see Note 31).
 4. Prepare lung, liver, and ear pinna for H&E analysis. Liver and ear can be directly placed into tissue cassettes for processing. Lungs must be inflated with 10% neutral buffered formalin (see Note 32) prior to placing in cassettes. To inflate lungs, place the mouse on its back and dissect away skin on the front side of mouse from mid section to head. Make a small incision through the muscle, just below the neck, revealing the trachea. Using a 5-ml syringe fitted with a 25-G needle filled with 10% formalin, gently insert needle into trachea. Slowly fill lungs with formalin solution until they are visibly expanded. Remove needle, excise lungs, and place in tissue cassette. All cassettes must be stored in 10% neutral buffered formalin to fix and preserve samples. Samples should be paraffin embedded, sectioned at 5 μ m, and stained with H&E, according to standard histological methods.
 5. Transfer spleens and lymph nodes into a 24-well cell culture plate containing 1 ml HBSS or PBS and maintain on ice at all times during processing.
 6. Process and assess mice individually. Tease apart each spleen and pooled set of lymph nodes separately with a 1-ml syringe plunger through a 70- μ m cell strainer into a 50-ml conical tube. Rinse cell strainer 2 times with HBSS to recover all cells. Alternatively, spleens and lymph nodes may be teased apart between two frosted glass microscope slides.
 7. Centrifuge homogenate at $300 \times g$ for 10 min.
 8. Resuspend homogenate in 1 ml Gey's solution per spleen. Gently swirl for 2 min and then quench reaction by adding 12 ml of HBSS.
 9. Centrifuge at $300 \times g$ for 10 min.
 10. Resuspend cells in 1 ml of FACS buffer containing 10% normal mouse serum for 5 min on ice. Add an additional 1 ml of FACS buffer containing fluorescently conjugated anti-CD4 and anti-CD25 antibodies and incubate for 20 min on ice.
 11. Count cells by trypan blue exclusion using a hemocytometer.
 12. Centrifuge cells at $300 \times g$ for 2 min and wash twice with FACS buffer
 13. Resuspend cells in 100 μ l of FACS buffer and analyze by flow cytometry to determine the percentages of total CD4⁺ and CD4⁺ CD25⁺ T cells.

³⁰In most cases, IACUC approval must be obtained to photograph animals.

³¹For cell count consistency and accurate comparison between treated and untreated groups, we routinely take 6 lymph nodes: 2 inguinal, 2 axillary, and 2 cervical. Additional lymph nodes can be taken if so desired.

14. Calculate the number of total CD4⁺ and CD4⁺ CD25⁺ T cells by calculating the total number of live cells in the spleen or lymph nodes multiplied by the percentage of total CD4⁺ and CD4⁺ CD25⁺ T cells determined by flow cytometry.

3.7.4. Microscopic Analysis and Histological Scoring—The severity of inflammation should be assessed and scored in a blinded manner by an experienced veterinary pathologist. The scoring system used for assessing inflammation in *Foxp3*[−] mice is based on a simple algorithm for expressing inflammatory infiltrates in the lungs, liver, and ear. The scores allotted to these three tissues were 0–9, 0–11, and 0–8, respectively, giving a maximum possible total of 28.

Lung: The lumen of the airways and alveoli do not have any inflammatory infiltrates. However, in *Foxp3*[−] mice lacking T_{regs}, inflammation is present surrounding the bronchioles, the pulmonary blood vessels, and with expansion of the interstitium (septa) (Fig. 3). Each of these is separately assigned a numerical number of 0, 1, 2, or 3. A score of 0 is assigned if minimal or no inflammatory infiltrates are associated with the interstitium, the tissue surrounding the bronchioles and the blood vessels. A score of 1, 2, or 3 is assigned if inflammation is associated with <10%, 10–50%, or >50% of the bronchioles, blood vessels, or interstitium, respectively.

Liver: Portal tract inflammation, periportal/periseptal interface hepatitis, and hepatic lobular inflammatory foci are the three criteria for scoring the degree of inflammation in the liver of *Foxp3*[−] mice lacking T_{regs} (Fig. 3). Portal inflammation is scored 0–3 while interface hepatitis and lobular inflammatory foci are allotted a score of 0–4. *Portal inflammation*: A score of 0 is assigned when portal tracts do not have any inflammatory cells. A score of 1, 2, or 3 is assigned if inflammation is associated with <25, 25–75, or >75% of the liver portal tracts, respectively. *Periportal/periseptal interface hepatitis*: A focus of interface hepatitis associated with either a few or most of the portal tracts are scored 1 and 2, respectively. Two or more foci of interface hepatitis surrounding <50 or >50% of the portal tracts or perisepatae is scored 3 and 4, respectively. *Lobular inflammation*: Foci of granulocytes and/or lymphocytes with or without necrotic hepatocytes that expand the sinusoid are considered foci of inflammation while foci of granulocytes without necrotic hepatocytes that do not expand the sinusoid are excluded as foci of extramedullary hematopoiesis. The number of inflammatory foci in 10 contiguous 10× objective fields are counted and recorded as the average number of foci per 10× field and given a score of 0–4. A score of 0 is assigned when sinusoidal foci of inflammatory cells are absent. One focus or less per 10× field, 2–4 foci per 10× field, 5–10 foci per 10× field, and more than ten foci per 10× field are scored 1, 2, 3, and 4, respectively.

Ear pinna: The percent of the ear dermis with inflammatory infiltrates and the intensity of the dermal inflammation are the criteria for determining the degree and severity of the ear involved with inflammation in *Foxp3*[−] mice lacking T_{regs} (Fig. 3). *Percent of ear with inflammation*: The ear specimen is divided into equal linear segments. The average percent of an inflammatory cell infiltrate of all the segments is scored 0, 1, 2, 3, or 4. A score of 0 is assigned when the inflammatory cells in all segments are not beyond that of normal background level. A score of 1, 2, 3, or 4 is assigned when the average percent for the segments is <25, 25–50, 51–75, or >75%, respectively. *Intensity of inflammation*: The intensity of the inflammatory infiltrate in the dermis is assessed as being of a loose or dense nature. A score of 0 is assigned when inflammatory cells in the dermis are not beyond the normal background level. The intensity of the inflammation is considered loose when the majority of the inflammatory cells do not abut one another. The inflammation intensity is considered dense when the majority of the inflammatory cells abut one another. When all the inflammation is of the loose nature, a score of 1 is assigned. When there is a mixture of

loose and dense inflammatory cell infiltrates, a score of two is assigned when the loose form is dominant; A score of three is assigned when the dense form is dominant; A score of 4 is assigned when all of the inflammation is of a dense nature.

Histological scoring parameters:

1. Lung

a. Peribronchiolar inflammation.

- Score 0** Minimal or no inflammation.
- Score 1** <10% of bronchioles with inflammation.
- Score 2** 10–50% of bronchioles with inflammation.
- Score 3** >50% of bronchioles with inflammation.

b. Perivascular inflammation.

- Score 0** Minimal or no inflammation.
- Score 1** <10% of blood vessel with inflammation.
- Score 2** 10–50% of blood vessel with inflammation.
- Score 3** >50% of blood vessel with inflammation.

c. Interstitium.

- Score 0** Minimal or no inflammation.
- Score 1** <10% of interstitium with inflammation.
- Score 2** 10–50% of interstitium with inflammation.
- Score 3** >50% of interstitium with inflammation.

2. Liver

a. Portal tract inflammation.

- Score 0** Portal tracts with minimal or no inflammation.
- Score 1** <25% of tracts with inflammation.
- Score 2** 25–75% of tracts with inflammation.
- Score 3** >75% of tracts with inflammation.

b. Portal/periseptal interface hepatitis.

- Score 0** Portal tracts with minimal or no inflammation.
- Score 1** A few tracts with a focus of interface hepatitis.
- Score 2** Most tracts with a focus of interface hepatitis.
- Score 3** <50% of tracts with multiple foci of interface hepatitis.
- Score 4** >50% of tracts with multiple foci of interface hepatitis.

c. Hepatitis lobular inflammation.

- Score 0** Minimal or no parenchymal inflammatory cells.

- Score 1** One focus or less per 10x field.
- Score 2** Two to four foci per 10x field.
- Score 3** Five to ten foci per 10x field.
- Score 4** More than ten foci per 10x field.

3. Ear pinna

a. Percent of ear with inflammation.

- Score 0** Inflammatory cells not beyond background level.
- Score 1** <25% of dermis with inflammation.
- Score 2** 25–50% of dermis with inflammation.
- Score 3** 51–75% of dermis with inflammation.
- Score 4** >75% of dermis with inflammation.

b. Intensity of inflammation in the ear.

- Score 0** Inflammatory cells not beyond background level.
- Score 1** Inflammatory cells have loose arrangement.
- Score 2** Inflammatory cells have a loose and dense arrangement with the former dominating.
- Score 3** Inflammatory cells have a loose and dense arrangement with the latter dominating.
- Score 4** Only dense arrangement of inflammatory cells.

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References

1. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *The Journal of Experimental Medicine* 1998;188:287–296. [PubMed: 9670041]
2. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *The Journal of Experimental Medicine* 1999;190:995–1004. [PubMed: 10510089]
3. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4(+) CD25(+) T cells with regulatory properties from human blood. *The Journal of Experimental Medicine* 2001;193:1303–1310. [PubMed: 11390437]

4. Belkaid Y. Regulatory T cells and infection: a dangerous necessity. *Nature Reviews* 2007;7:875–888.
5. Tang Q, Bluestone JA. The Foxp3⁺ regulatory T cell: a jack of all trades, master of regulation. *Nature Immunology* 2008;9:239–244. [PubMed: 18285775]
6. Bettini M, Vignali DA. Regulatory T cells and inhibitory cytokines in autoimmunity. *Current Opinion in Immunology* 2009;21:612–618. [PubMed: 19854631]
7. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 2005;22:329–341. [PubMed: 15780990]
8. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DA. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 2007;450:566–569. [PubMed: 18033300]
9. Workman CJ, Vignali DA. Negative regulation of T cell homeostasis by lymphocyte activation gene-3 (CD223). *Journal of Immunology* 2005;174:688–695.
10. Goldrath AW, Bogatzki LY, Bevan MJ. Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. *The Journal of Experimental Medicine* 2000;192:557–564. [PubMed: 10952725]
11. Cho BK, Rao VP, Ge Q, Eisen HN, Chen J. Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells. *The Journal of Experimental Medicine* 2000;192:549–556. [PubMed: 10952724]
12. Allez M, Mayer L. Regulatory T cells: peace keepers in the gut. *Inflammatory Bowel Diseases* 2004;10:666–676. [PubMed: 15472533]
13. Maloy KJ, Salaun L, Cahill R, Dougan G, Saunders NJ, Powrie F. CD4⁺CD25⁺ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. *The Journal of Experimental Medicine* 2003;197:111–119. [PubMed: 12515818]
14. Powrie F, Leach MW, Mauze S, Menon S, Caddle LB, Coffman RL. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4⁺ T cells. *Immunity* 1994;1:553–562. [PubMed: 7600284]
15. Mottet C, Uhlig HH, Powrie F. Cutting edge: cure of colitis by CD4⁺CD25⁺ regulatory T cells. *Journal of Immunology* 2003;170:3939–3943.
16. Cahill RJ, Foltz CJ, Fox JG, Dangler CA, Powrie F, Schauer DB. Inflammatory bowel disease: an immunity-mediated condition triggered by bacterial infection with *Helicobacter hepaticus*. *Infection and Immunity* 1997;65:3126–3131. [PubMed: 9234764]
17. Yen D, Cheung J, Scheerens H, Poulet F, McClanahan T, McKenzie B, Kleinschek MA, Owyang A, Mattson J, Blumenschein W, Murphy E, Sathe M, Cua DJ, Kastelein RA, Rennick D. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *The Journal of Clinical Investigation* 2006;116:1310–1316. [PubMed: 16670770]
18. Kohm AP, Carpentier PA, Anger HA, Miller SD. Cutting edge: CD4⁺CD25⁺ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *Journal of Immunology* 2002;169:4712–4716.
19. Selvaraj RK, Geiger TL. Mitigation of experimental allergic encephalomyelitis by TGF-beta induced Foxp3⁺ regulatory T lymphocytes through the induction of anergy and infectious tolerance. *Journal of Immunology* 2008;180:2830–2838.
20. Jager A, Dardalhon V, Sobel RA, Bettelli E, Kuchroo VK. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *Journal of Immunology* 2009;183:7169–7177.
21. O'Connor RA, Prendergast CT, Sabatos CA, Lau CW, Leech MD, Wraith DC, Anderton SM. Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. *Journal of Immunology* 2008;181:3750–3754.
22. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *The Journal Of Experimental Medicine* 2005;201:233–240. [PubMed: 15657292]

23. Seliger B, Wollscheid U, Momburg F, Blankenstein T, Huber C. Characterization of the major histocompatibility complex class I deficiencies in B16 melanoma cells. *Cancer Research* 2001;61:1095–1099. [PubMed: 11221838]
24. Fidler IJ. Selection of successive tumour lines for metastasis. *Nature: New Biology* 1973;242:148–149.
25. Poste G, Doll J, Hart IR, Fidler IJ. In vitro selection of murine B16 melanoma variants with enhanced tissue-invasive properties. *Cancer Research* 1980;40:1636–1644. [PubMed: 7370995]
26. Turk MJ, Guevara-Patino JA, Rizzuto GA, Engelhorn ME, Sakaguchi S, Houghton AN. Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. *The Journal of Experimental Medicine* 2004;200:771–782. [PubMed: 15381730]
27. Zhang P, Cote AL, de Vries VC, Usherwood EJ, Turk MJ. Induction of postsurgical tumor immunity and T-cell memory by a poorly immunogenic tumor. *Cancer Research* 2007;67:6468–6476. [PubMed: 17616708]
28. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F. Disruption of a new fork-head/winged-helix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature Genetics* 2001;27:68–73. [PubMed: 11138001]
29. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+regulatory T cells. *Nature Immunology* 2003;4:330–336. [PubMed: 12612578]
30. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, Tanoue T, Imaoka A, Itoh K, Takeda K, Umesaki Y, Honda K, Littman DR. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 2009;139:485–498. [PubMed: 19836068]
31. Gaboriau-Routhiau V, Rakotobe S, Lecuyer E, Mulder I, Lan A, Bridonneau C, Rochet V, Pisi A, De Paepe M, Brandi G, Eberl G, Snel J, Kelly D, Cerf-Bensussan N. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. *Immunity* 2009;31:677–689. [PubMed: 19833089]

Fig. 1.

Gating profile for sorting T_{regs} and T_{conv} cells. The cells are first gated on live lymphocytes (not shown) and then a second gate is placed on the CD4⁺ cells (histogram). The CD4⁺ cells are further separated into either a CD45RB^{high}/CD25⁻ (T_{conv}) gate or CD45RB^{low}/CD25⁺ (T_{reg}) gate.

Fig. 2.

Procedures and analysis pertaining to the B16 melanoma and *Foxp3*⁻ rescue models. **(a)** The area on the flank of the mice that requires shaving and the location of the i.d. injection for the B16 melanoma model. **(b)** Sexing of a female (*left*) and male (*right*). **(c)** The proper technique recommended to hold a 2-day-old pup for i.p. injections. **(d)** The i.p. injection technique for a 2-day-old pup in the *Foxp3*⁻ rescue model. **(e)** Exterior of *Foxp3*⁻ (*left*) (*right*). mice that received no *T*_{regs} or wild-type natural *T*_{regs} **(f)** Spleens and lymph nodes of *Foxp3*⁻ (*left*) or wild-type mice that received no *T*_{regs} natural *T*_{regs} (*right*). Black bar, 1 cm

Fig. 3.

Microscopic H&E illustrations of *Foxp3*⁺ and *Foxp3*⁻ littermate mice of the lung, liver and ear pinna. (*Lung*) The *Foxp3*⁺ mouse does not have inflammatory cells in or around either the bronchioles (B) or blood vessels (BV), and the interstitial septae (IS) are narrow, thin, and lack inflammatory cell infiltrates. In the *Foxp3*⁻ mouse, inflammatory cell infiltrates (*) surround bronchioles (B) and pulmonary blood vessels (PV) and focally thicken the interstitial septae (IS). (*Liver*) The portal tracts (T) and liver lobular parenchyma (P) of the *Foxp3*⁺ mouse lack inflammatory infiltrates. Inflammatory cells fill some portal tracts (T) of the *Foxp3*⁻ mouse and they infiltrate the periportal hepatocytes broadening the portal tracts consistent with interface hepatitis. Foci of inflammatory cells (*) are randomly scattered through the liver lobular parenchyma of the *Foxp3*⁻ mouse. (*Ear pinna*) The dermis (D) and fatty (F) tissue of the *Foxp3*⁺ are void of inflammatory cells. Inflammatory cell infiltrates are present in a loose (*) and dense (**) pattern. The dermis is thicker and the fat tissue is obscured with inflammatory cell infiltrates in the *Foxp3*⁻ mouse.

Overview of five in vivo T_{reg} suppression models

Table 1

Model	Target cells ^a	Resources required ^b	Time to results ^c	Time requirements ^d	Technical procedures ^e	Technical complexity ^f
Homeostasis	Naïve homeostatically expanding CD4 ⁺ T cells	Minimal	7 days	Minimal	i.v. Injections	Simple
IBD Recovery	Th1 T cells (Th17)	Moderate/substantial (large number of recipient mice and significant access to sort facilities on demand)	56 days	Weekly monitoring and weighing Frequent monitoring upon sickness Detailed analysis and histology	i.v. Injections i.p. Injections Optional mucosal analysis Histological analysis	Moderate
EAE	Th17 and Th1 T cells	Moderate (model-specific reagents including peptides, adjuvants, and toxins)	30 days	6 injections Daily monitoring	Emulsions s.c. Injections i.p. Injections	Moderate
B16 Melanoma	CD8 ⁺ T cells	Substantial (large number of mice, significant amount of sorting and many model-specific reagents including inoculation and surgical reagents)	1 ^o tumor: 15–20 days 2 ^o tumor: additional 15–20 days	Daily monitoring of tumors Multiple injections Multihour surgery	i.v. Injections i.d. Injections Measurement of tumors Surgical resection of tumors Isolation of tumor infiltrating lymphocytes	Difficult
<i>Foxp3</i> [−] rescue	Primarily lymphocytes	Moderate (large number of <i>Foxp3</i> [−] breeders, moderate number of donor mice and access to sort facilities on demand)	25–30 days	Timed/monitored pregnancies Long sorts Difficult injections Time consuming analysis	Marking/ genotyping 1-day-old pups i.p. Injections into 2-day-old pups Histological analysis	Moderate

^aThe cell populations that are primarily suppressed by T_{regs} in the model listed

^bAn indication of the amount of mice, sort time, and materials required for an average 3 group experiment as described in the methods

^cTime required to complete one experiment starting from the initial injections of the mice. Time required for analysis is not included and will be in addition to time noted

^dStages in the protocols that may be time demanding

^eProcedures that are required in the protocol that may require some level of training depending upon the investigator's level of expertise. This does not include sorting and flow cytometry, which are required techniques in all of the models

^fThe overall level of complexity for each protocol, taking into consideration time, resources, and techniques required