Video Article

Mouse Naïve CD4$^+$ T Cell Isolation and *In vitro* Differentiation into T Cell Subsets

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Abstract

Antigen inexperienced (naïve) CD4$^+$ T cells undergo expansion and differentiation to effector subsets at the time of T cell receptor (TCR) recognition of cognate antigen presented on MHC class II. The cytokine signals present in the environment at the time of TCR activation are a major factor in determining the effector fate of a naïve CD4$^+$ T cell. Although the cytokine environment during naïve T cell activation may be complex and involve both redundant and opposing signals *in vivo*, the addition of various cytokine combinations during naive CD4$^+$ T cell activation *in vitro* can readily promote the establishment of effector T helper lineages with hallmark cytokine and transcription factor expression. Such differentiation experiments are commonly used as a first step for the evaluation of targets believed to promote or inhibit the development of certain CD4$^+$ T helper subsets. The addition of mediators, such as signaling agonists, antagonists, or other cytokines, during the differentiation process can also be used to study the influence of a particular target on T cell differentiation. Here, we describe a basic protocol for the isolation of naïve T cells from mouse and the subsequent steps necessary for polarizing naïve cells to various T helper effector lineages *in vitro*.

Video Link

The video component of this article can be found at http://www.jove.com/video/52739/

Introduction

The concept of distinct lineages or subsets of CD4$^+$ T helper (Th) cells has been around since the latter part of the 20th century[1]. Recognition of cognate antigen in the presence of costimulatory signals results in several rounds of cellular proliferation and the eventual differentiation into effector Th cells. The type of Th cell generated during this process is dependent on the cytokine environment present during activation[2]. Initially, naïve Th cells were thought to polarize into 2 distinct lineages following T cell receptor (TCR) activation, costimulatory CD28 ligation, and cytokine signaling. Type 1 helper cells (Th1) are characterized by their effector production of the IFNγ cytokine as well as their requirement for IL-12 signaling during the differentiation process[3,4]. Eventually it was discovered that differentiated Th1 cells have a genetic profile that is most distinctively characterized by the expression of the T box family transcription factor, *Tbx21* (T-bet), which is considered the master regulator of the Th1 genetic program[5]. Furthermore, IL-12 as well as IFNγ can promote T-bet expression[6,7]. In the immune response, Th1 cells are important for the host defense against intracellular pathogens as well as strong promoters of autoimmune inflammation. In contrast, type 2 helper cells (Th2) require IL-4 for their development and their effector cytokines, including IL-4, IL-5, and IL-13, are important for driving B cell responses and are pathogenic in allergy[8,9]. Similar to Th1 cells, Th2 cells were found to express their own master transcriptional regulator, termed GATA-3[10,11]. Interestingly, the presence of polarizing cytokines and the generation of a specific Th lineage are antagonistic to the development of others[2,12], suggesting that only a particular Th subset may become dominant during an immune response.

Since the identification of the Th1 and Th2 lineages, further work has demonstrated even more unique subsets of T helper cells, including follicular helper (TFH), IL-9-producing (Th9), and IL-22-producing (Th22)(recently reviewed in[13]). For the purposes of *in vitro* differentiation experiments, this protocol will focus only on two additional Th subsets, termed regulatory T cells (Treg) and IL-17-producing CD4$^+$ T cells (Th17). CD25$^+$ regulatory T cells can occur naturally (nTreg) in the thymus; naïve Th cells may also be induced (iTreg) to become regulatory in the periphery (reviewed in[14,15]). Both types of Tregs express a characteristic transcription factor, termed forkhead box P3 (Foxp3), which is critical for their effector suppression mechanisms that include soluble anti-inflammatory mediator production, IL-2 consumption, and cell contact-dependent mechanisms[14,15]. The lack of Foxp3 expression results in a severe, multi-organ autoimmune disorder termed immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), demonstrating the critical role of this Th subset in resolving inflammation and regulating peripheral tolerance to self antigens[16]. In vitro, naïve CD4$^+$ T helper cells up-regulate Foxp3 and become committed to the Treg program upon stimulation with IL-2 and TGF-β[14,15]. There may be moderate to considerable plasticity in CD4$^+$ T cell lineages, especially when considering only cytokine production (reviewed in [17,18]). However, for the purposes of *in vitro* differentiation protocols, we will be discussing each subset as a unique lineage.

Recently, a subset of Th17 cells that produces the IL-17 cytokine was identified as a unique lineage with pro-inflammatory functions that are particularly pathogenic during autoimmune inflammation[19-21]. Th17 cells express a unique transcription factor, termed retinoid-related orphan

receptor gamma t (RORγt) that coordinates the Th17 genetic program[22]. TGFβ is important for the generation of Th17 lineage through the induction of RORγt. However, the effect of TGFβ signaling is believed to only induce Th17 commitment upon synergizing with IL-6 (reviewed in[12]). Further studies have shown that a variety of other signals that can positively regulate Th17 commitment, including IL-1β, increased sodium, and TLR signaling[23-26]. Other reports have suggested that the pathogenic Th17 cells *in vivo* are the ones that actually bypass TGFβ signaling and instead rely on a combination of IL-1, IL-6, and IL-23 for their differentiation[27]. Thus, Th17 cells may be derived from a variety of signaling pathways; for the purposes of this protocol, the commonly-used (TGFβ and IL-6) pathway for Th17 lineage commitment will be presented.

The differentiation protocols described below for all effector lineages relies on fixed antibody as stimuli for the TCR and CD28 throughout the entire course of the experiment. However, others have demonstrated that TCR activation with antigen-presenting cells[28] or cross-linking anti-CD3 and anti-CD28 antibodies with hamster antibody for 2 days[29] are also highly effective means of inducing the generation of various Th subsets. The protocol presented here builds on previously reported methods for isolating murine $CD4^+$ T cells from secondary lymphoid organs[30] and generating Th17 cells[31]. One major difference is that this protocol relies on the use of a cell sorter to isolate naïve $CD4^+$ T cells from lymphoid tissues. However, many companies now offer rapid separation kits that can enrich for naïve $CD4^+$ T cells, which may be able to bypass the requirement for sorting depending on the experiment. The methods and reagents presented in this protocol are what we routinely use and find to be the most effective. However, keep in mind that alternative reagents and methodologies exist for many of the steps presented below and it is up to the individual lab to determine what will work best for their purposes.

Protocol

All experimental procedures are performed using protocols approved by the office of Environmental Health and Safety at the Rosalind Franklin University of Medicine and Science. C57BL/6 mice (purchased from NCI) used for this protocol were housed under specific pathogen-free conditions, and all animal experiments were performed using protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the Rosalind Franklin University of Medicine and Science.

1. Preparation of Instruments, Supplies, and Reagents

1. Coat 48-well or 24-well plates with anti-CD3 and anti-CD28 (**Table 1**) in sterile PBS, cover and wrap in parafilm to prevent evaporation and contamination, and then incubate O/N at 4 ^{o}C the day before T cell isolation. Alternatively, coat wells 1 h at 37 ^{o}C. Perform Th0, Th1, Th2, and iTreg conditions by coating both anti-CD3 and anti-CD28 at 1μg/ml in 0.5-1 ml total volume (48-well plate). For Th17, we typically coat anti-CD3 at 2 μg/ml and anti-CD28 at 1 μg/ml.
   NOTE: We have found that a lower anti-CD3 concentration is optimal for Th1, Th2, and iTreg generation while a higher anti-CD3 concentration is optimal for Th17 generation. Thus, anti-CD3 and anti-CD28 concentrations should be titrated and optimized for individual laboratories (**Table 2**).
2. Sterilize a set of dissection tools (scissors and forceps) by autoclave or disinfectant solution. A tube or small beaker containing 70% ethanol will be needed for quick sterilization of tools between dissections. Finally, a spray bottle containing 70% ethanol will be needed for sterilizing the surface of the mouse before dissection.
3. Prepare a dissection surface by wrapping a flat piece of Styrofoam or corkboard in aluminum foil. Dissecting pins or needles are used to fix the animal in place.
4. Prepare dishes or plates for the collection of tissues. If pooling tissues, separate 60mm dishes containing 3ml of sterile PBS + 1% FBS (PBS +) for spleen and lymph node tissues is sufficient. If keeping tissues from individual mice separate, prepare a 24-well plate containing 1ml of PBS+ in the desired number of wells.
5. Prepare complete RPMI media for cell culture and pre-warm before use (**Table 1**).
   NOTE: We routinely use RPMI but other media such as IMDM and DMEM may be equally effective or superior depending on the lab and the experiment.
6. Cut 2x2 cm squares of nylon mesh with a 120 μm pore size and autoclave to sterilize (**Table 1**).
7. Optional: if using the high-speed magnetic cell sorting system such as autoMACS for $CD4^+$ cell enrichment, pre-warm running and rinsing buffers in a 37 ^{o}C water bath to prevent damage to the machine.
8. NOTE: Enrichment is recommended to increase the sorting yield and decrease sorting time.
9. All preparation steps, except for dissection, should be performed in a sterile tissue culture hood.

2. Isolation of Lymph Nodes and Spleen from Mice

1. Euthanize the required number of animals using an institutionally-approved technique. Use C57BL/6 female mice, age 5-10 wks, due to their high percentage of naïve T cells and lower body fat in comparison to males or to older mice.
   NOTE: Cells derived from male mice will perform similarly *in vitro* and the protocol steps remain the same. Female mice, 5-10 wks old, will typically yield 3-6 x 10^6 naïve $CD4^+$ T cells per mouse. However, using different strains of mice may affect yield and performance. For example, cells from C57BL/6 mice are better for generating Th1 cells while cells from Balb.c mice are better for generating Th2 cells.
2. Spread the limbs of the animal and pin them in place as shown in **Figure 1**. Cut the skin longitudinally from the anus to the chin while being careful not to puncture deeper tissue.
3. Spread open the skin with forceps and pin the skin in place to allow easy access to lymph nodes as shown in **Figure 1**.
4. Harvest the accessible lymph nodes from the locations shown in **Figure 1**. Grabbing the lymph node with a pair of forceps while separating tissue with another pair of forceps will allow for easy removal. Place the tissue in the collection dish.
5. Harvest the spleen from the location shown in **Figure 1**. In this case, careful cutting into the peritoneum is needed to gain access to the spleen. Place the tissue in a collection dish.
6. Repeat the procedure if more tissues will be harvested. Otherwise proceed to the next steps. At this point, perform all remaining steps on ice until the plating of the T cells after sorting.

3. Tissue Processing and $CD4^+$ Enrichment

1. Process the lymph nodes and spleen in different dishes to increase the yield as lymph node cell preparations do not require erythrocyte lysis, which may result in minor leukocyte death. Therefore, the following steps describe a method for processing splenic and lymph node populations separately followed by combining prior to labeling for sorting.
2. Remove the tissue (spleen or pooled lymph nodes) from the collection dish and place in a new 60 mm dish containing 3 ml of PBS+. Place a square of sterile nylon mesh over the tissue using sterilized forceps.
3. Take the thumb side of a syringe plunger (3-10 ml) and gently smash the tissue against the mesh. Almost all of the tissue should go into suspension. Alternatively, place the tissue between two autoclaved frosted slides and grinded into suspension.
4. Pipette the suspension up and down a few times to break up the remaining soluble clumps. Place a new square piece of nylon mesh over the opening of a 15 ml conical tube and filter the suspension through to remove the remaining debris.
5. Repeat steps 2-4 for additional lymph node and splenic tissue.
6. Once the suspension has been filtered into a 15ml tube fill the remainder of the tube with PBS+ and invert a few times. Centrifuge the cells at 475 x g for 5 min at 4 ^oC.
7. For the splenic cells, aspirate the supernatant following centrifugation and resuspend the cell pellet in 1 ml of ice-cold 1X ACK solution per spleen for 1 min to lyse erythrocytes. Then add 10 ml of PBS+ on top of the ACK, invert the tube, and centrifuge 475 x g for 5 min at 4 ^oC.
8. For lymph node cells, aspirate the supernatant and resuspend in 2ml of PBS+. If desired these can be combined with the splenic cells after their ACK lysis and washing step.
9. Aspirate the supernatant after centrifugation of the splenocytes and resuspend in another 10 ml of PBS+. At this point the lymph node suspension can be added on top of the spleen suspension if tissue pooling is required for sorting. Centrifuge the tube again at 475 x g for 5 min at 4 ^oC.
10. The cell pellet can now be prepared for CD4 enrichment. If this step will not be performed, proceed to the sorting preparation steps.
11. For CD4 enrichment using the high-speed magnetic cell sorting system, resuspend the cellular pellet with CD4 beads. Typically 15 µl of beads diluted in 85 µl of PBS+ is used to label the tissues from 1 mouse. Ramp up the volume of bead mixture as needed, resuspend the pellet, and incubate for 15-30 min at 4 ^oC.
12. Wash the cells with 10 ml of PBS+, pass the suspension through nylon into a new 15 ml tube to remove debris, and centrifuge again at 475 x g for 5 min at 4 ^oC.
13. Resuspend the pellet in 100 µl of PBS+ per mouse and proceed to positive selection on the high-speed magnetic cell sorting system. If using a manual separation system, follow the manufacturer's instructions for enrichment. Alternatively, resuspend the samples in FACS buffer: PBS with 1mM EDTA (pH = 8) and 1% BSA, sterile-filtered.
14. Remove the positive fraction and wash again with 10ml of PBS+. The enriched $CD4^+$ fraction is now ready to be labeled for sorting.

4. Sorting Naïve $CD4^+$ T cells

1. Label the cell pellet with PBS+ containing fluorescent antibodies directed against CD62L, CD44, CD25, and CD4. If using the antibodies listed in **Table 1**, dilutions for each are provided. For staining, a 100 µl volume of the antibody cocktail is used per tissues from one mouse.
2. Resuspend the pellet in antibody cocktail and incubate for 15-30 min at 4 ^oC in the dark.
3. Wash the cells with 10ml of PBS+ and centrifuge 475 x g for 5 min at 4 ^oC.
4. Pass the mixture over another nylon filter or cell strainer cap to remove any leftover debris. Pipette the labeled cells into a tube that is compatible for the cell sorter. Keep cells on ice and protected from the light until the sort.
5. Prepare collection tubes by pipetting 1-2ml of complete RPMI media or FBS into the bottom of tubes compatible for collection on a cell sorter.
6. Sort the naïve $CD4^+$ T cells as a $CD4^+CD25^-CD62L^+CD44^-$ population (**Figure 2**). Wash the collected cells with complete RPMI media and centrifuge as described above.
7. Resuspend the pellet in complete RPMI, count the naïve cells, and adjust the concentration to 1 x 10^6 cells/ml.

5. Setting Up the *In vitro* Differentiation

1. Aspirate the wells of the anti-CD3 and anti-CD28 coated plates and wash each well with 1ml of sterile PBS (no FBS). For 48-well plates, plate 0.5 x 10^6 cells/well in 0.5 ml of RPMI. For 24-well plates, culture 1 x 10^6 cells/well in 1.0 ml of RPMI.
2. If testing the influence of a factor such as a drug add the substance to the appropriate wells either before, during, or after the differentiation process depending on the experiment. Next, supplement the culture media with cytokines and blocking antibodies accordingly. Th0: 30 U/ml hIL-2. Th1: 15 ng/ml rmIL-12, 30 U/ml hIL-2, and 5,000 ng/ml 11B11.Th2: 10 ng/ml rmIL-4, 30 U/ml hIL-2, 2,000 ng/ml soluble 37.51, and 5,000 ng/ml XMG1.2. iTreg: 15 ng/ml hTGFβ, 30 U/ml hIL-2, 5,000 ng/ml XMG1.2, and 5,000 ng/ml 11B11. Th17: 20 ng/ml rmIL-6, 3 ng/ml hTGFβ, 5,000 ng/ml XMG1.2, and 5,000 ng/ml 11B11.
   NOTE: The conditions presented above were found to be optimal for maximizing cytokine production as measured at the end of the differentiation experiment (*Representative Results* section). However, each condition should be optimized for individual laboratories (**Table 2**). Furthermore, the addition of soluble anti-CD28 in addition to the plate-bound anti-CD28 has been found to enhance Th2 cytokine production in our laboratory, but has no effect on other T cell subsets:
3. Incubate the plate at 37 ^oC with 5% CO_2 for 4-5 d prior to analysis of gene expression and cytokine production. Alternatively, remove the cells from the TCR stimuli after 2 d, adjusted to 1 x 10^6 cells/ml with fresh media, and plate in new wells (non-coated) to enhance proliferation and final yield. In this case, fresh polarizing cytokines and antibodies are not needed but 10 U/ml of IL-2 should be added to new media for Th0, Th1, Th2, and iTreg cultures.

6. Analysis of Differentiation

1. For cytokine analysis by flow cytometry, restimulate each well with 10ng/ml PMA and 1,000 ng/ml ionomycin or 1 µg/ml anti-CD3 for 4-6 hr in the presence of a golgi inhibitor such as brefeldin A (**Table 1**). Perform intracellular cytokine staining depending on the experiment (**Figure 3**).

NOTE: Stain non-lineage-specific cytokines or transcription factors, such as IL-4 (not shown) and IFNγ (**Figure 3**) for Th17 cultures, for each condition as negative controls and as an indicator of differentiation efficiency. Furthermore, stain Foxp3 for Th17 cultures as the reciprocal development of Tregs may occur with the addition of TGFβ.

2. For protein quantification, collect the supernatants from each well and assay by cytokine-specific ELISA (**Figure 3**). If there is concern that the factor being tested has influenced proliferation compared to control samples, the cells can be washed, counted, normalized, and then restimulated with 1 µg/ml of anti-CD3 for 24 h before collecting supernatants for ELISA assays. If assaying IL-4 from Th2 conditions, washing and restimulating is necessary to remove IL-4 that may be leftover from the initial differentiation culture.
3. For gene expression analysis by real time PCR, harvest cells after the incubation period, wash, normalize, and re-stimulate for 2-6 h with 1 µg/ml of anti-CD3 prior to harvesting mRNA (**Figure 3**).

Representative Results

The time point for the analysis of differentiation can vary depending on the Th condition being tested as well as the strength of T cell receptor activation. After 2-3 days of differentiation, cells can be visualized by light microscopy to determine the extent of T cell proliferation. Wells exhibiting extensive proliferation and clumping of cells will most likely be ready for analysis at day 4. Differentiation conditions relying on the addition of exogenous IL-2, such as Th1 and Th2, will likely exhaust the media after 4 days in culture. To maximize cytokine production, such wells with exhausted media can be replenished with fresh RPMI containing IL-2 or counted and re-plated at 1 x 10^6 cells per well in fresh RPMI containing IL-2 to extend the differentiation for another day or two using this method.

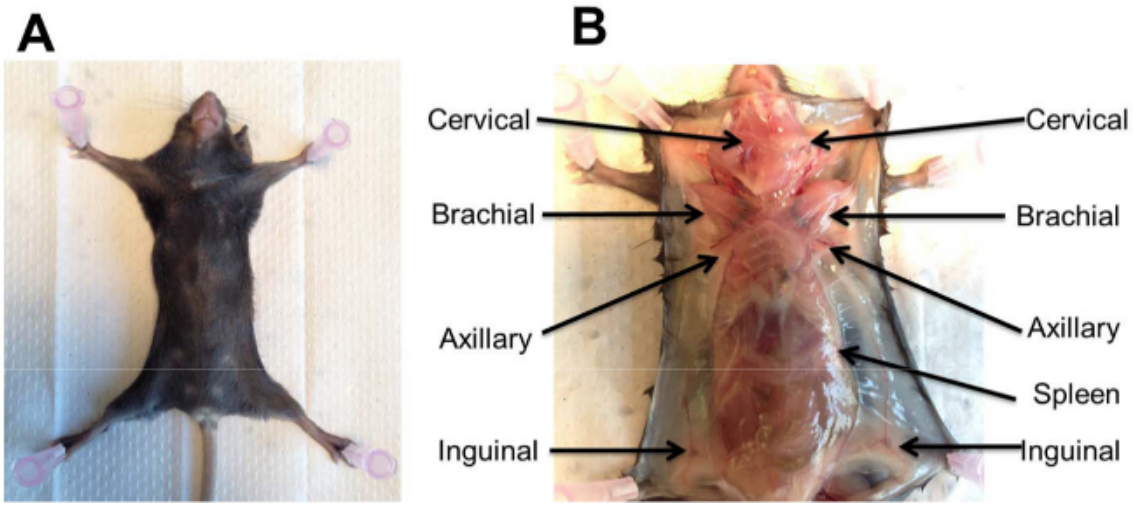

Figure 1. Illustration of pin placement to fix a mouse for the isolation of spleen and lymph nodes. (A) Spread the limbs apart and fix them with a sterile needle or dissection pin. From this position, cut the fur and skin longitudinally from the tail to the chin. **(B)** After cutting, gently spread the skin apart to expose the lymph nodes as shown in the picture. The locations of lymph nodes that are easily accessible from this position are listed in the picture. The spleen is located just under the ribcage on the right side of the animal. Please click here to view a larger version of this figure.

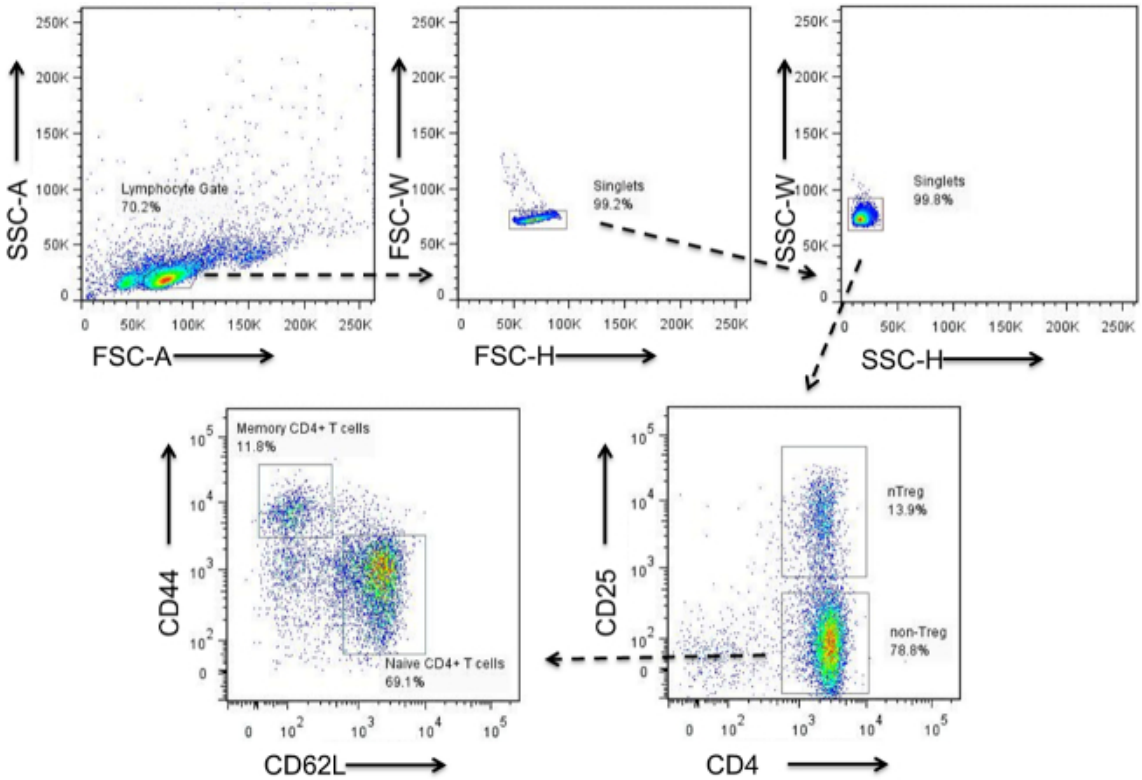

Figure 2. Representative sorting strategy for the isolation of naïve CD4$^+$ T cells by cell sorting. Starting from the upper left, lymphocytes are first gated by size and then singlet cells are discriminated from doublet cells. Gating on singlets reveals a population of CD4$^+$CD25$^+$ cells that are nTregs. Gate on the CD4$^+$CD25$^-$ population and then sort naïve (CD62L$^+$CD44$^-$) from the effector/memory (CD62L$^-$CD44$^+$) CD4$^+$ cells. Dotted lines indicate the gate origin of the next plot. Please click here to view a larger version of this figure.

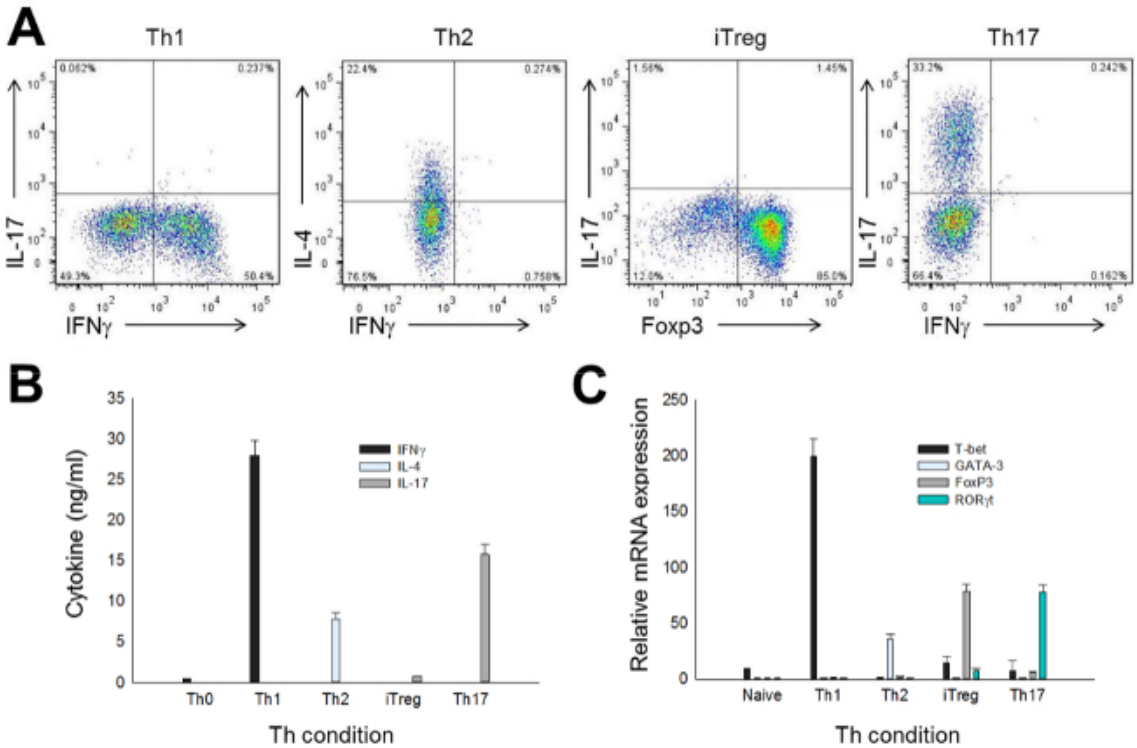

Figure 3. Sample data following *in vitro* Th differentiation. (A) Intracellular cytokine staining data (gated on $CD4^+$) from Th1, Th2, iTreg, and Th17 differentiations. Typically, non-lineage specific cytokines, such as IL-4 and IFNγ for Th17 cultures, should be stained to determine the quality of the differentiation. **(B)** ELISA data following 4 d Th differentiation, washing, and 24 h restimulation of cells with 1 µg/ml anti-CD3. **(C)** Real time PCR data of lineage-specific transcription factors following 4 d differentiation and restimulation for 4 h with 1 µg/ml anti-CD3. All gene quantities were normalized to the expression of β-actin and mRNA from undifferentiated naïve T cells serve as the baseline for gene expression. Please click here to view a larger version of this figure.

Table 1. See Materials Table below.

Reagent	Recommended Titration Range
Cytokines:	
Human (h) IL-2	5 U/ml - 50 U/ml
rmIL-4	5 ng/ml - 30 ng/ml
rmIL-6	5 ng/ml - 30 ng/ml
rmIL-12	10 ng/ml - 30 ng/ml
hTGFb (Th17)	0.1 ng/ml - 5 ng/ml
hTGFb (iTreg)	5 ng/ml - 30 ng/ml
Antibodies:	
2C11 (anti-CD3)	500 ng/ml - 5,000 ng/ml
37.51 (anti-CD28)	250 ng/ml - 2,500 ng/ml
11B11 (anti-IL-4)	5,000 - 10,000 ng/ml
XMG1.2 (anti-IFNg)	5,000 - 10,000 ng/ml

Table 2. Recommended Titration Range for the Optimization of Differentiation Conditions.

Discussion

While the spleen contains naïve Th cells, the proportion of this population in lymph nodes is much higher. Failure to properly identify and remove lymph nodes in this protocol will result in a poor yield of naïve cells. This can be especially difficult in older mice or male mice that have more fat tissue. As shown in **Figure 1**, proper fixing and pinning of the animal limbs and skin will allow for easier visualization of the accessible exterior lymph nodes. Once lymph nodes and spleens are processed, cell sorting is the preferred method of obtaining a highly purified naïve $CD4^+$ T cell population. Purity is critical to prevent nTregs or effector and memory Th cells from influencing the naïve cell differentiation process. Difficulties

in obtaining a high yield of naïve T cells are typically caused by inefficient tissue collection and processing or loss of cells during the separation steps. To increase yield make sure to process the cells using cold buffers and incubations on ice. Furthermore, to prevent loss of cells during enrichment and/or sorting, optimizing the reagents and separation protocols beforehand is recommended. Finally, as mentioned at various points in the protocol steps, differentiation conditions should be optimized for individual laboratories before performing large scale experiments (**Table 2**).

As shown in **Figure 2**, naïve $CD4^+$ T cells can be easily be distinguished from the nTreg ($CD4^+CD25^+$) and effector ($CD4^+CD44^+CD62L^-$) populations. Sorting is one of the most reliable ways to prevent contamination of effector Th subsets, which also exist in the spleen and lymph nodes. Treg contamination during the differentiation may result in the suppression of activation as well as cytokine production. Contamination of other effector Th subsets may result in the production of cytokines that are inhibitory to the development of desired Th lineages. Sorting facility fees can be expensive and some find that it is easier to enrich for $CD4^+$ T cells by magnetic separation to cut down on sorting time as described in this protocol. In the absence of a sorter, some companies now offer separation kits designed specifically for the isolation of naïve $CD4^+$ T cells. However, the purity of the population should always be checked after separation, similar to sorted samples. The finding of a low frequency of naïve $CD4^+$ T cells ($CD62L^+$) in comparison to the memory/effector cells ($CD44^-$) may indicate a problem with the mouse, such as an infection or tumor. In this case the cells should not be used for this type of experiment.

There are quite a few ways to determine the effectiveness of Th differentiation. Protein analysis is traditionally performed by cytokine staining, which will give an idea of the percentage of naïve cells that have been polarized to a specific lineage (**Figure 3**). Protein quantification by ELISA is also a useful tool to determine the total amount of cytokines being released into the media. The caveat with ELISA protein analysis though is that comparing results between experimental groups may be difficult if a particular factor being tested has an influence on proliferation and survival. In this case, the total numbers of cells producing cytokines may be different between groups at the end of the experiment. Thus, normalizing the cell count and restimulating for a short period of time (*e.g.*, anti-CD3 for 24 h) is necessary to obtain appropriate results. Furthermore, IL-4 is used as a polarizing factor for the generation of the Th2 subset, so these cells must be washed, normalized, and restimulated prior to performing an ELISA for IL-4. Gene expression analysis by real time PCR is useful not only for the analysis of cytokine gene transcription, but also for lineage-specific transcription factors (**Figure 3**). Thus, depending on the experiment, there are various ways to assay the effectiveness of Th differentiation.

Disclosures

The authors declare no competing financial interests.

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References

1. Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., Coffman, R. L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol.* **136**, (7), 2348-2357 (1986).
2. Dong, C., Flavell, R. A. Cell fate decision: T-helper 1 and 2 subsets in immune responses. *Arthritis Res.* **2**, (3), 179-188 (2000).
3. Hsieh, C. S., *et al.* Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science.* **260**, (5107), 547-549 (1993).
4. Seder, R. A., Gazzinelli, R., Sher, A., Paul, W. E. Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. *Proc Natl Acad Sci U S A.* **90**, (21), 10188-10192 (1993).
5. Szabo, S. J., *et al.* A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell.* **100**, (6), 655-669 (2000).
6. Afkarian, M., *et al.* T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4. *T cells. Nat Immunol.* **3**, (6), 549-557 (2002).
7. Zhu, J., *et al.* The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. *Immunity.* **37**, (4), 660-673 (2012).
8. Le Gros, G., Ben-Sasson, S. Z., Seder, R., Finkelman, F. D., Paul, W. E. Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *J Exp Med.* **172**, (3), 921-929 (1990).
9. Swain, S. L., Weinberg, A. D., English, M., Huston, G. IL-4 directs the development of Th2-like helper effectors. *J Immunol.* **145**, (11), 3796-3806 (1990).
10. Zhang, D. H., Cohn, L., Ray, P., Bottomly, K., Ray, A. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *J Biol Chem.* **272**, (34), 21597-21603 (1997).
11. Zheng, W., Flavell, R. A. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell.* **89**, (4), 587-596 (1997).
12. Dong, C. TH17 cells in development: an updated view of their molecular identity and genetic programming. *Nat Rev Immunol.* **8**, (5), 337-348 (2008).
13. Jiang, S., Dong, C. A complex issue on CD4(+) T-cell subsets. *Immunol Rev.* **252**, (1), 5-11 (2013).
14. Rudensky, A. Y. Regulatory T cells and Foxp3. *Immunol Rev.* **241**, (1), 260-268 (2011).
15. Sakaguchi, S., Yamaguchi, T., Nomura, T., Ono, M. Regulatory T cells and immune tolerance. *Cell.* **133**, (5), 775-787 (2008).
16. Barzaghi, F., Passerini, L., Bacchetta, R. Immune dysregulation, polyendocrinopathy, enteropathy, x-linked syndrome: a paradigm of immunodeficiency with autoimmunity. *Front Immunol.* **3**, 211 (2012).
17. Shea, J. J., Paul, W. E. Mechanisms underlying lineage commitment and plasticity of helper CD4 T cells. *Science.* **327**, (5969), 1098-1102 (2010).

18. Zhou, L., Chong, M. M., Littman, D. R. Plasticity of CD4+ T cell lineage differentiation. *Immunity.* **30**, (5), 646-655 (2009).
19. Harrington, L. E., *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol.* **6**, (11), 1123-1132 (2005).
20. Langrish, C. L., *et al.* IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med.* **201**, (2), 233-240 (2005).
21. Park, H., *et al.* A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol.* **6**, (11), 1133-1141 (2005).
22. Ivanov, I. I., *et al.* The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell.* **126**, (6), 1121-1133 (2006).
23. Chung, Y., *et al.* Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity.* **30**, (4), 576-587 (2009).
24. Reynolds, J. M., Martinez, G. J., Chung, Y., Dong, C. Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation. *Proc Natl Acad Sci U S A.* **109**, (32), 13064-13069 (2012).
25. Reynolds, J. M., *et al.* Toll-like receptor 2 signaling in CD4(+) T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease. *Immunity.* **32**, (5), 692-702 (2010).
26. Wu, C., *et al.* Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. *Nature.* **496**, (7446), 513-517 (2013).
27. Ghoreschi, K., *et al.* Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. *Nature.* **467**, (7318), 967-971 (2010).
28. Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M., Stockinger, B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity.* **24**, (2), 179-189 (2006).
29. Avni, O., *et al.* T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nat Immunol.* **3**, (7), 643-651 (2002).
30. Matheu, M. P., Cahalan, M. D. Isolation of CD4+ T cells from mouse lymph nodes using Miltenyi MACS purification. *J Vis Exp.* (9), (2007).
31. Bedoya, S. K., Wilson, T. D., Collins, E. L., Lau, K., Larkin, J. 3rd Isolation and th17 differentiation of naive CD4 T lymphocytes. *J Vis Exp.* (79), e50765 (2013).

