Journal of Visualized Experiments

Induction of Intestinal Inflammation by Adoptive Transfer of CBir1 TCR Transgenic CD4+ T Cells to Immunodeficient Mice

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE63293R2
Full Title:	Induction of Intestinal Inflammation by Adoptive Transfer of CBir1 TCR Transgenic CD4+ T Cells to Immunodeficient Mice
Corresponding Author:	Yingzi Cong University of Texas Medical Branch galveston, TX UNITED STATES
Corresponding Author's Institution:	University of Texas Medical Branch
Corresponding Author E-Mail:	yicong@utmb.edu
Order of Authors:	Wenjing Yang
	Tianming Yu
	Yingzi Cong
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Medicine
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Galveston, Texas, United States
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release
Please provide any comments to the journal here.	

TITLE:

Induction of Intestinal Inflammation by Adoptive Transfer of CBir1 TCR Transgenic CD4$^+$ T Cells to Immunodeficient Mice

AUTHORS AND AFFILIATIONS:

Wenjing Yang[1], Tianming Yu[1], Yingzi Cong[1]*

[1]Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, 77555, USA

Email addresses of the authors:

Wenjing Yang (wenyang@utmb.edu)
Tianming Yu (tiayu@utmb.edu)
Yingzi Cong (yicong@utmb.edu)

*Email address of the corresponding author:

Yingzi Cong (yicong@utmb.edu)

SUMMARY:

In this protocol, a gut microbiota antigen-specific T cell adoptive transfer colitis model is described. CD4$^+$ T cells are isolated from CBir1 TCR transgenic mice. These are specific for an immunodominant gut microbiota antigen CBir1 flagellin, which is transferred into recipient *Rag1*$^{-/-}$ mice, leading to intestinal inflammation.

ABSTRACT:

With the increase of incidence, inflammatory bowel diseases (IBD), which are chronic diseases affecting the gastrointestinal tract, impose a considerable health and financial burden on individuals and society. Therefore, it is critical to investigate the mechanisms underlying the pathogenesis and development of IBD. Here, a gut microbiota antigen-specific T cell transfer colitis model is described. CBir1 flagellin has been recognized as the immunodominant gut bacterial antigen in experimental colitis and patients with Crohn's disease. CBir1 TCR transgenic naïve CD4$^+$ T cells, specific to CBir1 flagellin, can induce chronic colitis after adoptive transfer into immune-deficient *Rag1*$^{-/-}$ mice. The disease severity is assessed by histopathology. The CD4$^+$ T cell phenotypes in colonic lamina propria are also determined. This model closely resembles the development of IBD, which provides an ideal murine model for investigating the mechanisms driving the pathogenesis of IBD and testing the potential drugs for treating IBD.

INTRODUCTION:

Inflammatory bowel diseases (IBD), mainly including Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic, relapsing-remitting inflammation of the gastrointestinal tract, affecting millions worldwide[1]. Several factors have been implicated in the development and pathogenesis of IBD, including genetic susceptibility, gut microbiota, immune responses, diet, and lifestyle[2]. However, the exact mechanism of IBD is still not completely understood.

One of the particular interests is the interaction between gut microbiota and host immune responses in regulating intestinal inflammation[3]. Gut microbiota provides a series of immunostimulatory molecules and antigens, which can activate immune responses[4]. While the balance between effector T cells and regulatory T cells (Tregs) is critical in maintaining intestinal homeostasis, the excessive intestinal mucosal $CD4^+$ T cell response to gut microbiota antigens contributes to intestinal inflammation[5-7]. As an immunodominant gut microbiota antigen, CBir1 flagellin has been related to the pathogenesis of human CD[8,9]. Furthermore, transfer of CBir1 TCR transgenic (Tg) T cells induces intestinal inflammation in immune-deficient mice[6], closely resembling the human IBD, indicating that this T cell transfer model helps investigate the mechanisms of human IBD.

This work describes the detailed protocol of inducing colitis in *Rag1*$^{-/-}$ mice by adoptive transfer of CBir1 TCR Tg naïve $CD4^+$ T cells and assessing disease severity. Besides, the anticipated results are shown, and the critical steps of the procedure and troubleshooting are discussed, which will help researchers investigate the mechanisms of pathogenesis of intestinal inflammation and test the potential drugs for treating IBD.

PROTOCOL:

All animal procedures were performed according to the University of Texas Medical Branch's Committee on the Use and Care of the animals. CBir1 TCR Tg mice were provided by Dr. Charles Elson of the University of Alabama at Birmingham. CBir1 TCR Tg mice can be female or male but should be at 8-12 weeks. *Rag1*$^{-/-}$ mice on the C57BL/6 background were obtained from the Jackson Laboratory[10]. *Rag1*$^{-/-}$ mice must be gender and age-matched, and either male or female can be used but should be at 8-12 weeks. The entire protocol is summarized in **Figure 1**.

1. Preparation of the recipient mice

1.1 Prepare *Rag1*$^{-/-}$ mice on the C57BL/6 background, bred in the same specific pathogen-free animal facility. Calculate the number of mice per group by power analysis[11].

NOTE: *Rag1*$^{-/-}$ mice do not have mature T cells and B cells[10].

1.2 Mark the mice by ear punch.

1.3 Weigh the mice on the same day of T cell transfer.

2. Preparation of the reagents and solutions

NOTE: The reagents used are toxic, or biohazard and their handling need precautions and safety measures.

2.1 Prepare Washing Buffer: add 5 mL of 100x Penicillin-Streptomycin into 500 mL of RPMI 1640 medium. Mix it thoroughly and store it at 4 °C.

2.2 Prepare Tris-NH_4Cl Lysis Buffer.

2.2.1 Prepare Solution Part A. Dissolve 2.06 g of Tris base in 100 mL of double-distilled water (ddH_2O) and adjust the pH value to 7.2 with HCl.

2.2.2 Prepare Solution Part B. Dissolve 7.47 g of NH_4Cl in 800 mL of ddH_2O.

2.2.3 Mix A and B thoroughly. Measure the pH and adjust it to 7.2 if not.

2.2.4 Adjust the total volume to 1000 mL. Autoclave and then store it at 4 °C.

2.3 Prepare the Isolation Buffer.

2.3.1 Add 2.5 g of BSA and 500 µL of EDTA (0.5 M, pH 8.0) into 500 mL of 1x PBS. Mix it thoroughly.

2.3.2 Filter the solution through a 0.22 µm vacuum-driven disposable bottle top filter (see **Table of Materials**). Store it at 4 °C.

2.4 Prepare FACS Buffer. Add 1 mL of FBS and 50 µL of EDTA (0.5 M, pH 8.0) into 50 mL of Washing Buffer (prepared in step 2.1). Mix thoroughly and store it at 4 °C.

2.5 Prepare Complete Medium. Add 5 mL of FBS in 45 mL of Washing Buffer. Mix thoroughly and store it at 4 °C.

2.6 Prepare EDTA-PBS Buffer.

2.6.1 Calculate the volume of the EDTA-PBS Buffer needed. Volume (mL) = mouse number x 20.

2.6.2 Add appropriate volume of FBS, EDTA, and HEPES in the PBS (2% of FBS, 0.5 mM of EDTA, 10 mM of HEPES in PBS) (see **Table of Materials**).

2.6.3 Mix it thoroughly and pre-warm in a 37 °C water bath.

2.7 Prepare the Digestion Buffer.

2.7.1 Calculate the volume of the Digestion Buffer needed. Volume (mL) = mouse number x 20.

2.7.2 Add appropriate volume of FBS, Collagenase IV, and DNase I in Washing Buffer (2% of FBS, 0.5 mg/mL of Collagenase IV, and 10 U/mL of DNase I in Washing Buffer) (see **Table of Materials**).

2.7.3 Mix it thoroughly and pre-warm in a 37 °C water bath.

2.8 Prepare Percoll Solution.

2.8.1 Prepare 100% Percoll. Add 5 mL of 10x PBS in 45 mL of original Percoll (see **Table of Materials**).

2.8.2 Prepare 2% FBS in Washing Buffer. Add 1 mL of FBS in 49 mL of Washing Buffer.

2.8.3 Caulculate the volume of 40 % Percoll Solution and 75 % Percoll Solution. Volume of 40% Percoll Solution (mL) = mouse number x 4; Volume of 75% Percoll Solution (mL) = mouse number x 2.

NOTE: The reagents/solutions prepared in steps 2.1-2.5 will be used in steps 3-4, and those prepared in steps 2.6-2.8 will be used in step 9. All the reagents/solutions used in step 9 should be freshly prepared. Making 5 % extra Buffer is recommended for all the steps.

3. Isolation of splenic CBir1 TCR Tg CD4$^+$ T cells

3.1 Euthanize CBir1 TCR Tg mouse/mice by a cervical dislocation with CO_2 euthanasia (30%-70% gas-air displacement rate). Wet the mice with 70% ethanol.

3.2 Perform a ~1 cm left abdomen incision, pull the skin away from the abdominal muscle tissue, make a ~3 cm incision in the abdominal muscle tissue, and remove the spleen with sterile scissors and forceps. Place the spleen in a culture dish containing 5 mL of pre-cold Washing Buffer (prepared in step 2.1).

3.3 Grind the spleen with the rough surface of two sterile glass slides. Transfer the cell suspension into a 50 mL centrifuge tube by passing through a 100 µm cell strainer (see **Table of Materials**). Rinse the glass slides and culture dish with 5 mL of pre-cold Washing Buffer and transfer the Washing Buffer into the tube.

3.4 Centrifuge the cell suspension at 350 x *g* for 8 min at 4 °C. Discard the supernatant and resuspend the cells with 5 mL of pre-warmed Tris-NH_4Cl Lysis Buffer (prepared in step 2.2) per spleen. Incubate for 10 min at room temperature. Add 10 mL of pre-cold Washing Buffer to the tube.

3.5 Centrifuge the cell suspension at 350 x *g* for 8 min at 4 °C. Discard the supernatant and resuspend the cells with 10 mL of pre-cold Isolation Buffer (prepared in step 2.3).

3.6 Count the cells. Mix 10 µL of cell suspension with 10 µL of trypan blue thoroughly. Load 10 µL of the mixture onto a slide, insert the slide into the Automated Cell Counter (see **Table of Materials**) and obtain the viable cell number[12].

NOTE: Approximately 1 x 10^8 cells can be obtained from one donor mouse in this step.

3.7 Centrifuge the remaining cell suspension from step 3.6 at 350 x *g* for 8 min at 4 °C. Discard all the supernatants.

3.8 Vortex the anti-mouse CD4 Magnetic Particles thoroughly (see **Table of Materials**), directly add 50 µL of the particles per 10^7 cells and mix with cell pellets thoroughly. Incubate for 30 min at 4 °C.

NOTE: Any other commercial $CD4^+$ T cell enrichment kit can be used here.

3.9 Transfer the cell-particle suspension into a sterile collection tube. Add 3.5 mL of pre-cold Isolation Buffer into the tube.

3.10 Place the tube on the Cell Separation Magnet (see **Table of Materials**) for 8 min at room temperature. Carefully aspirate off the supernatant using a 3 mL Transfer Pipette.

3.11 Remove the tube from Cell Separation Magnet (see **Table of Materials**), resuspend the cells with 3.5 mL pre-cold Isolation Buffer, and place the tube to the Magnet for 4 min at room temperature. Carefully aspirate off the supernatant using a 3 mL Transfer Pipette.

3.12 Repeat step 3.11.

3.13 Resuspend the cells with 1 mL of pre-cold FACS Buffer (prepared in step 2.4).

4. Purification of CBir1 TCR Tg naïve $CD4^+$ T cells

4.1 Count the cells following step 3.6.

4.2 If the cell concentration is >10^7/mL, add a volume of FACS buffer to make sure the cell concentration is ≤ 10^7/mL.

NOTE: ~1 × 10^7 cells can be obtained from one donor mouse in this step.

4.3 Stain the surface markers with 10 µL of anti-mouse CD4-APC, 10 µL of anti-mouse CD25-Percp/Cy5.5, and 10 µL of anti-mouse CD62L-PE[13,14] (see **Table of Materials**). Mix gently and incubate for 30 min at 4 °C in the dark.

4.4 Wash the cells with 2 mL of pre-cold FACS buffer. Centrifuge the cell suspension at 350 x *g* for 8 min at 4 °C. Aspirate all the supernatants using a 3 mL Transfer Pipette.

4.5 Repeat Step 4.4.

4.6 Resuspend the cells to the concentration of 40 x 10^6/mL in pre-cold FACS buffer.

NOTE: To prevent the sorter from clogging, pass the cells through a 70 µm strainer.

4.7 Add 0.1 µg/mL of DAPI.

NOTE: DAPI is used for excluding the dead cells.

4.8 Prepare 15 mL centrifuge tubes containing 4 mL of Complete Medium (prepared in step 2.5) for collecting the sorted cells.

4.9 Load the cells onto the sorter. Sort single viable naïve $CD4^+$ T cells ($DAPI^-$ $CD4^+$ $CD25^-$ $CD62L^+$ cells) in purity mode (Nozzle size: 70 µm; Pressure: 70 PSI; Event rate: 8000-12000 events/s; Efficiency: higher than 90%) (**Figure 2**).

NOTE: Naïve $CD4^+$ T cells express high expression of CD62L and lack the activation marker CD25[13,14].

4.10 Centrifuge the cell suspension at 350 x *g* for 8 min at 4 °C. Aspirate all the supernatants using a 3 mL Transfer Pipette.

4.11 Resuspend the cells in 500 µL of 1x PBS.

4.12 Count cells following step 3.6

NOTE: ~5×10^6 cells can be obtained from one donor mouse in this step.

5. Cell transfer into the recipient mice

5.1 Resuspend the CBir1 TCR Tg naïve $CD4^+$ T cells to the 5 x 10^6/mL concentration by adding 1x PBS.

5.2 Warm the *Rag*$^{-/-}$ mice under a heat lamp (see **Table of Materials**) for 4 min, and restrain the mice using a mouse restrainer.

5.3 Intravenously transfer 200 µL of the cell suspension into the tail vein of *Rag*$^{-/-}$ mice using a 1 mL insulin syringe (27 G) (see **Table of Materials**).

NOTE: Cells from one donor mouse are enough to transfer to around five recipient mice.

6. Monitoring clinical signs during colitis progression

6.1 Weigh the mice every week and increase the observation to twice a week once the mice start losing >5% of original weights.

6.2 Observe mice response/move when gently stimulated.

6.3 Observe other clinical abnormalities. i.e., posture and stool consistency.

7. Colon collection and histopathological scoring

7.1 Sacrifice the recipient mice by a cervical dislocation with CO_2 at a time point of the weight loss >20% of original weight or 6-weeks post cell transfer. Wet the mice with 70% ethanol.

7.2 Perform a ~1 cm ventral midline skin incision, pull the skin away from the abdominal muscle tissue, make a ~3 cm incision in the abdominal muscle tissue, identify the cecum, and remove the entire colon with sterile scissors and forceps. Wet the colon with pre-cold PBS in a culture dish.

7.3 Incise the colon lengthwise and rinse it with pre-cold PBS. Cut 1/3 of the colon longitudinally.

7.4 Place the colon strip in a paper towel with the luminal side facing upward. Perform Swiss rolling using a toothpick[15].

7.5 Place the colon Swiss into a cassette and put the cassette in 10% buffered formalin for 24 h[16], followed by dehydration and paraffin embedding using an automated processor (see **Table of Materials**).

7.6 Cut 5 µm tissue sections on a microtome, mounted on slides, and perform the Hematoxylin and eosin (H&E) stain[17] (see **Table of Materials**).

7.7 Determine the histopathological scores by combining the scores for each of the six parameters for a maximum of 12. Lamina propria inflammation (normal, 0; mild, 1; moderate, 2; severe, 3); goblet cell loss (normal, 0; mild, 1; moderate, 2; severe, 3); abnormal crypt (normal, 0; hyperplastic, 1; disorganization, 2; crypt loss, 3); crypt abscesses (absent, 0; present, 1); mucosal erosion and ulceration (normal, 0; mild, 1; moderate, 2; severe, 3); and submucosal change (none, 0; submucosa, 1; transmural, 2)[18].

8. Isolation and staining of intestinal lamina propria cells

8.1. After step 7.3, cut another 2/3 of the colon into 0.5-1 cm pieces and wash it with pre-cold PBS.

8.2. Transfer the colon segments into 20 mL pre-warmed EDTA-PBS buffer in a 50 mL centrifuge tube. Incubate at 37 °C with 250 rpm shaking for 30 min.

8.3. Vortex the tube, discard the supernatants by passing it through a sterile sieve (diameter: 0.01 inches), and resuspend the colon segments in 20 mL of pre-cold PBS in the 50 mL tube.

8.4. Repeat step 8.3 twice.

8.5. Place the colon segments in a C tube (see **Table of Materials**) containing 10 mL of pre-warmed Digestion Buffer.

8.6. Place the tube on a Dissociator machine (see **Table of Materials**) and incubate under the program of "37C_m_LPDK_1" for 25 min.

NOTE: "37C_m_LPDK_1" is a standard preset program in the Dissociator machine used for stirring the samples and keeping them at 37 °C.

8.7. Check if tissue is digested completely, which means that no piece of tissue is in the Digestion buffer. If not, repeat the program of "37C_m_LPDK_1".

8.8. Collect the supernatant by passing through a metal sieve and 100 µL strainer. Rinse with 10 mL of pre-cold PBS.

8.9. Centrifuge the cell suspension at 350 x *g* for 8 min at 4 °C. Aspirate all the supernatants.

8.10. Resuspend the cells in 4 mL of 40% Percoll Solution and mix it thoroughly (prepared in step 2.8).

8.11. Transfer the resuspended cells to 2 mL of 75% Percoll solution in a 15 mL centrifuge tube.

8.12. Centrifuge the cell suspension at 850 x *g* for 20 min at 20 °C (Acceleration ramp: 0; Brake ramp: 0).

8.13. Carefully remove fat on the top layer using a 3 mL Transfer Pipette and transfer the cell layer to 20 mL of Washing Buffer in a 50 mL tube.

8.14. Centrifuge the cell suspension at 350 x g for 8 min at 4 °C. Discard all the supernatants and resuspend cells in 1 mL of Completed Medium.

8.15. Count the cells following step 3.6.

8.16. Seed the cells in a 24-well plate, activate them with 50 ng/mL of Phorbol-12-myristate 13-acetate and 750 ng/mL of ionomycin for 2 h, followed by incubation with 5 µg/mL of Brefeldin A for 3 h (see **Table of Materials**).

NOTE: The reagents used are toxic, and their handling needs precautions and safety measures.

8.17. Transfer the cells into a FACS tube, add 2 mL of FACS Buffer, and centrifuge the cells at 350 x *g* for 8 min at 4 °C. Discard the supernatant.

8.18. Incubate the cells with 12.5 µg/mL of anti-mouse CD16/32[19] in (see **Table of Materials**) FACS buffer to block Fc receptors for 5 min at room temperature.

8.19. Stain for live/dead and surface marker.

8.19.1. Wash the cells with 2 mL of FACS Buffer and centrifuge them at 350 x *g* for 8 min at 4 °C. Discard the supernatant.

8.19.2. Stain the cells with live dye and surface markers (i.e., anti-mouse CD3 and anti-mouse CD4 antibodies)[20] (see **Table of Materials**) in FACS Buffer at the optimized concentration for 30 min at 4 °C in the dark.

NOTE: The reagents used are toxic, and their handling needs precautions and safety measures.

8.20. Perform the cellular and nuclear staining.

8.20.1. Add 2 mL of FACS Buffer and centrifuge the cell suspension at 350 x *g* for 8 min at 4 °C. Discard the supernatant.

8.20.2. Permeabilize and fix the cells by resuspending the cells with 200 µL of Transcription Factor Fix working solution (see **Table of Materials**) for 40 min at room temperature.

8.20.3. Add 2 mL of Perm buffer and centrifuge the cell suspension at 350 x *g* for 8 min at 4 °C. Discard the supernatant.

8.20.4. Incubate the cells with cellular and nuclear markers (i.e., anti-mouse IFNγ, anti-mouse IL-17A, and anti-mouse Foxp3 antibodies)[20] in Perm buffer (see **Table of Materials**) for 30 min-1 h at room temperature.

8.20.5. Add 2 mL of Perm buffer and centrifuge the cell suspension at 350 x *g* for 8 min at 4 °C. Discard the supernatant.

8.20.6. Add 1 mL of FACS Buffer and centrifuge the cell suspension at 350 x g for 8 min at 4 °C. Discard the supernatant.

8.21. Resuspend cells with 200 µL of FACS Buffer and run the samples on a flow cytometer (see **Table of Materials**).

REPRESENTATIVE RESULTS:

Approximately 5 x 10^6 CBir1 TCR Tg naïve $CD4^+$ T cells per spleen were isolated from an adult CBir1 TCR Tg mouse. Transfer of CBir1 TCR Tg naïve $CD4^+$ T cells induced chronic colitis in recipient *Rag1*$^{-/-}$ mice. After cell transfer, clinical signs were monitored to evaluate the progression of intestinal inflammation, including weight loss, stool consistency, and hunched posture. As expected, mice began to lose weight around three weeks post cell transfer, and the weight

reached around 80%-85% of original weight six weeks post cell transfer (**Figure 3**). Additionally, mice showed diarrhea around 3-4 weeks post cell transfer and demonstrated hunched posture when they developed severe colitis. Gross morphology of the colon was shown, and the colitis severity was assessed by the histopathological score when mice were sacrificed. Mice receiving CBir1 TCR Tg naïve $CD4^+$ T cells showed short colon length 6 weeks post cell transfer (**Figure 4A**). The recipient mice demonstrated more cell infiltration in the intestinal lamina propria 4 weeks post cell transfer (**Figure 4C**), goblet cell loss and intestinal epithelial cell hyperplasia 5 weeks post cell transfer (**Figure 4D**), and mucosal erosion and inflammatory cell infiltration in the submucosa of the colon 6 weeks post cell transfer (**Figure 4F**). At the same time, there was no inflammation in *Rag1*$^{-/-}$ mice receiving PBS alone (**Figure 4B**). Besides, there is no inflammation in the small intestine, but the cecum has inflammation. In addition, $CD4^+$ T cell phenotypes in colonic lamina propria were determined by flow cytometry. The gating strategy is shown (**Figure 5A-E**). CBir1 TCR Tg naïve $CD4^+$ T cells developed into $IFN\gamma^+$ Th1 cells, IL-$17A^+$ Th17 cells, $IFN\gamma^+$ IL-17^+ $CD4^+$ T cells (**Figure 5F**), and $Foxp3^+$ Treg cells in intestinal lamina propria of *Rag*$^{-/-}$ recipients (**Figure 5G**).

FIGURE LEGENDS:

Figure 1: The procedure of induction colitis and assessment of disease severity. Splenic $CD4^+$ T cells were isolated from CBir1 TCR transgenic mice using magnetic beads, and then naïve T cells were purified by sorting. CBir1 TCR transgenic naïve T cells were then intravenously transferred into recipient *Rag1*$^{-/-}$ mice. When the mice were sacrificed around six weeks post cell transfer, colitis severity was assessed by histopathological scores. The $CD4^+$ T cell phenotypes in colonic lamina propria were determined by flow cytometry.

Figure 2: The gating strategy for sorting CBir1 TCR transgenic naïve T cells. Viable single CBir1 TCR transgenic naïve T cells were purified by excluding debris (**A**), non-single cells (**B-C**), dead cells (**D**), and activated cells (**E-F**). The subpopulation was shown in (**G**).

Figure 3: The weight changes of *Rag1*$^{-/-}$ mice post T cell transfer. 1 x 10^6 CBir1 TCR transgenic naïve T cells were transferred into *Rag1*$^{-/-}$ mice, and *Rag1*$^{-/-}$ mice receiving PBS were used as controls. Mouse weights were recorded every week. Data were presented in mean ± SD; Student's t-test; ***p<0.001.

Figure 4: The gross morphology and Hematoxylin and eosin staining of the colon from *Rag1*$^{-/-}$ mice receiving CBir1 TCR transgenic naïve T cells. (**A**) The recipient mice were sacrificed six weeks post cell transfer, and the gross morphology of the colon was shown. (**B-E**) The recipient mice were sacrificed at different time points, and *Rag1*$^{-/-}$ mice received PBS as controls. The colons were processed for Hematoxylin and eosin staining. Representative images of Hematoxylin and eosin staining of the colon are shown. Scale bar = 200 μm. (**F**) Histopathological scores were determined. Data were presented in mean ± SD.

Figure 5: The CD4+ T cell phenotypes in colonic lamina propria of *Rag*$^{-/-}$ mice receiving CBir1 TCR transgenic naïve T cells. When the recipient mice were sacrificed 6 weeks post cell transfer,

colonic lamina propria cells were isolated for staining $CD4^+$ T cell phenotypes. (**A-E**) The gating strategy for analysis of T cell phenotypes. (**F-G**) (**F**) IL-$17A^+$ $CD4^+$ T cells, $IFN\gamma^+$ $CD4^+$ T cells, IL-17^+ $IFN\gamma^+$ $CD4^+$ T cells, and (**G**) Foxp3+ Tregs were determined by flow cytometry.

DISCUSSION:

Although every step is essential for the reproducibility of this colitis model, there are several critical steps. The recipient *Rag*$^{-/-}$ mice should receive adequate viable naïve $CD4^+$ T cells to induce intestinal inflammation. We used spleens for the isolation of naïve $CD4^+$ T cells instead of MLNs. Because the yield of naïve $CD4^+$ T cells in MLNs is much lower than in spleens. CD62L is highly expressed in naïve T cells, and CD44 and CD25 are the activation markers of T cells[13,14]. In this study, we first used anti-CD4 magnetic beads to isolate $CD4^+$ T cells from spleens. Then we used the combination of anti-CD4, anti-CD62L, and anti-CD25 antibodies for isolation of naïve $CD4^+$ T cells[13,14]. Researchers could use other markers for sorting the naïve $CD4^+$ T cells. $CD45RB^{hi}$ is also marker of naïve T cells. $CD45RB^{hi}$ $CD25^-$ $CD4^+$ T cells are commonly used as naïve T cells of wild-type non-TCR transgenic T cells[23]. Therefore, the techniques of sorting cells and intravenously injection of the T cells into the recipient mice are essential. Setting up the gating protocol as a template is helpful to speed up the experiments with fewer errors. To avoid cell death, cells should always be kept on ice. Besides, staining cells with DAPI is highly recommended for excluding dead cells because DAPI cannot transit across intact cell membranes, making it an excellent dead cell probe[24]. Warming the mice to stimulate dilation of the tail veins provides better vein visibility for intravenous injection. All procedures are recommended to be performed by trained researchers.

Many factors might impact the outcome of the colitis models, which needs to be paid attention. First, the recipient *Rag1*$^{-/-}$ mice should be age and gender-matched. In the $CD45RB^{hi}$ T cell transfer model, T cells from male and female donors can be transferred to male *Rag*$^{-/-}$ recipients, while only female donors can be used when using female recipients[23]. However, we do not see a significant difference between male and female recipients. The recipient mice could be either females or males, and T cells from male donors can also induce colitis in female recipients. Since weight change is a valuable indicator of colitis progression, it is recommended to use the recipient mice between 8-12 weeks to present a stable weight line. These recipient mice should be bred and kept in the same room of the animal facility because microbiota is critical in regulating colitis development[25]. The time to develop colitis varies when transferring different numbers of CBir1 TCR Tg naïve CD4 T cells. As expected, fewer cells require a longer time for induction of colitis, and a higher number of cells require a shorter time for induction of colitis. Using 1 x 10^6 cells per recipient mouse is recommended since the recipient mice demonstrate clinical signs of colitis ~2-3 weeks post cell transfer and develop relatively severe colitis ~6 weeks post cell transfer. In addition, compared with intraperitoneal injection, intravenous injection of cells into the tail vein induces more consistent colitis. For isolation of colonic lamina propria cells, the colon tissues must not get dry; otherwise, it would reduce cells' yield and viability. One of the particular concerns is that the duration of the colitis would be changed if the recipient mice are transferred with genetic-modified CBir1 TCR Tg T cells or treated with drugs[26]. In addition, CBir1 TCR Tg T cells also induce intestinal inflammation in other immune-deficient mice, such as TCR$\beta/\delta^{-/-}$ mice, which lack T cells[27].

As accumulating evidence indicates a crucial role of gut bacterial antigen-specific reactive T cells in the pathogenesis of IBD, using T cells specific for a defined gut bacterial antigen will provide insights into how gut bacterial antigen induce T cell responses to induce colitis. Gut microbiota antigen CBir1 flagellin is abundant in the gastrointestinal tract, which is related to the pathogenesis of IBD[8,9]. This colitis model resembles several critical characteristics of IBD, including diarrhea, weight loss, histopathological finding, and abnormal intestinal immune responses. Therefore, this colitis model is useful to study the mechanisms of human IBD and provides a tool to evaluate the treatments for IBD. Interestingly, the recent work of Chiaranunt *et al.* indicated that T cell specificity to the microbiota CBir 1 antigen alone might not be sufficient to induce T cell activation and colitis. This is evidenced by the wild-type CBir1 TCR Tg T cells induced colitis in *Rag*$^{-/-}$ recipients, whereas *Rag*$^{-/-}$ CBir1 T cells did not induce colitis in their animal facility, suggesting that gut T cells responding to specific gut bacterial antigen may require other interrelated commensal bacteria, for example, *Helicobacter* spp, to function as an adjuvant[28]. An exciting aspect of this model is that different T cell subsets, namely Th1, Th17, and Treg cells, are present in lamina propria of colitic recipient mice, which provides a unique opportunity for investigating the roles of not only effector T cells but also Tregs in the pathogenesis of colitis[29].

However, as this colitis model is mediated by gut microbiota-specific T cells, one limitation for this colitis model is that the duration of induction of colitis may vary in different animal facilities depending on gut microbiota.

ACKNOWLEDGMENTS:

This work was supported in part by the National Institutes of Health grants DK125011, AI150210, and DK124132, the University of Texas System STARs award (Y.C.), and the James W. McLaughlin Fellowship Fund from The University of Texas Medical Branch at Galveston (W.Y.). Figure 1 was created with BioRender.com.

DISCLOSURES:

No authors have conflicting financial, professional, or personal interests.

REFERENCES:

1. Kaplan, G. G. The global burden of IBD: From 2015 to 2025. *Nature Reviews Gastroenterology & Hepatology.* **12** (12), 720-727 (2015).
2. Ananthakrishnan, A. N. Epidemiology and risk factors for IBD. *Nature Reviews Gastroenterology & Hepatology.* **12** (4), 205-217 (2015).
3. Yang, W., Cong, Y. Gut microbiota-derived metabolites in the regulation of host immune responses and immune-related inflammatory diseases. *Cellular & Molecular Immunology.* **18** (4), 866-877 (2021).
4. Pickard, J. M., Zeng, M. Y., Caruso, R., Núñez, G. Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. **279** (1), 70-89 (2017).
5. Russler-Germain, E. V., Rengarajan, S., Hsieh, C. S. Antigen-specific regulatory T-cell responses to intestinal microbiota. *Mucosal Immunology.* **10** (6), 1375-1386 (2017).
6. Chen, L. et al. Microbiota metabolite butyrate differentially regulates Th1 and Th17 cells'

differentiation and function in induction of colitis. *Inflammatory Bowel Diseases.* **25** (9), 1450-1461 (2019).
7. Cong, Y., Weaver, C. T., Lazenby, A., Elson, C. O. Bacterial-reactive T regulatory cells inhibit pathogenic immune responses to the enteric flora. *Journal of Immunology.* **169** (11), 6112-6119 (2002).
8. Lodes, M. J. et al. Bacterial flagellin is a dominant antigen in Crohn disease. *Journal of Clinical Investigation.* **113** (9), 1296-1306 (2004).
9. Targan, S. R. et al. Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease. *Gastroenterology.* **128** (7), 2020-2028 (2005).
10. Mombaerts, P. et al. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell.* **68** (5), 869-877 (1992).
11. Charan, J., Kantharia, N. D. How to calculate sample size in animal studies? *Journal of Pharmacology & Pharmacotherapeutics.* **4** (4), 303-306 (2013).
12. Kwizera, R. et al. Evaluation of trypan blue stain in the TC20 automated cell counter as a point-of-care for the enumeration of viable cryptococcal cells in cerebrospinal fluid. *Medical Mycology.* **56** (5), 559-564 (2018).
13. Boyman, O., Létourneau, S., Krieg, C., Sprent, J. Homeostatic proliferation and survival of naïve and memory T cells. *European Journal of Immunology.* **39** (8), 2088-2094 (2009).
14. Chai, J. G. et al. Regulatory T cells, derived from naïve CD4+CD25- T cells by in vitro Foxp3 gene transfer, can induce transplantation tolerance. *Transplantation.* **79** (10), 1310-1316 (2005).
15. Bialkowska, A. B., Ghaleb, A. M., Nandan, M. O., Yang, V. W. Improved Swiss-rolling technique for intestinal tissue preparation for immunohistochemical and immunofluorescent analyses. *Journal of Visualized Experiments.* **113**, e54161 (2016).
16. Au - Bialkowska, A. B., Au - Ghaleb, A. M., Au - Nandan, M. O., Au - Yang, V. W. Improved Swiss-rolling technique for intestinal tissue preparation for immunohistochemical and immunofluorescent analyses. *Journal of Visualized Experiments.* **113**, e54161 (2016).
17. Fischer, A. H., Jacobson, K. A., Rose, J., Zeller, R. Hematoxylin and eosin staining of tissue and cell sections. *Cold Spring Harbor Protocols*. **2008** (2008).
18. Erben, U. et al. A guide to histomorphological evaluation of intestinal inflammation in mouse models. *International Journal of Clinical and Experimental Pathology.* **7** (8), 4557-4576 (2014).
19. Tuijnman, W. B., Van Wichen, D. F., Schuurman, H. J. Tissue distribution of human IgG Fc receptors CD16, CD32 and CD64: An immunohistochemical study. *APMIS.* **101** (4), 319-329 (1993).
20. Yang, W. et al. Intestinal microbiota-derived short-chain fatty acids regulation of immune cell IL-22 production and gut immunity. **11** (1), 4457 (2020).
21. Reinoso Webb, C. *et al.* Differential susceptibility to t cell-induced colitis in mice: Role of the Intestinal Microbiota. *Inflammatory Bowel Disease.* **24** (2), 361-379 (2018).
22. Bamias, G. et al. Down-regulation of intestinal lymphocyte activation and Th1 cytokine production by antibiotic therapy in a murine model of Crohn's disease. *Journal of Immunology.* **169** (9), 5308-5314 (2002).
23. Steinbach, E. C., Gipson, G. R., Sheikh, S. Z. Induction of murine intestinal inflammation by adoptive transfer of effector CD4+ CD45RB high T cells into immunodeficient mice. *Journal of Visualized Experiments.* **98**, e52533 (2015).

24. Atale, N., Gupta, S., Yadav, U. C., Rani, V. Cell-death assessment by fluorescent and nonfluorescent cytosolic and nuclear staining techniques. *Journal of Microscopy.* **255** (1), 7-19 (2014).
25. Manichanh, C., Borruel, N., Casellas, F., Guarner, F. The gut microbiota in IBD. *Nature Reviews Gastroenterology & Hepatology.* **9** (10), 599-608 (2012).
26. Sun, M. et al. Microbiota-derived short-chain fatty acids promote Th1 cell IL-10 production to maintain intestinal homeostasis. *Nature Communications.* **9** (1), 3555 (2018).
27. Feng, T. et al. Th17 cells induce colitis and promote Th1 cell responses through IL-17 induction of innate IL-12 and IL-23 production. *Journal of Immunology.* **186** (11), 6313-6318 (2011).
28. Chiaranunt, P., Tometich, J. T. & Ji, J. T Cell Proliferation and Colitis Are Initiated by Defined Intestinal Microbes. **201** (1), 243-250 (2018).
29. Feng, T., Cao, A. T., Weaver, C. T., Elson, C. O., Cong, Y. Interleukin-12 converts Foxp3+ regulatory T cells to interferon-γ-producing Foxp3+ T cells that inhibit colitis. *Gastroenterology.* **140** (7), 2031-2043 (2011).

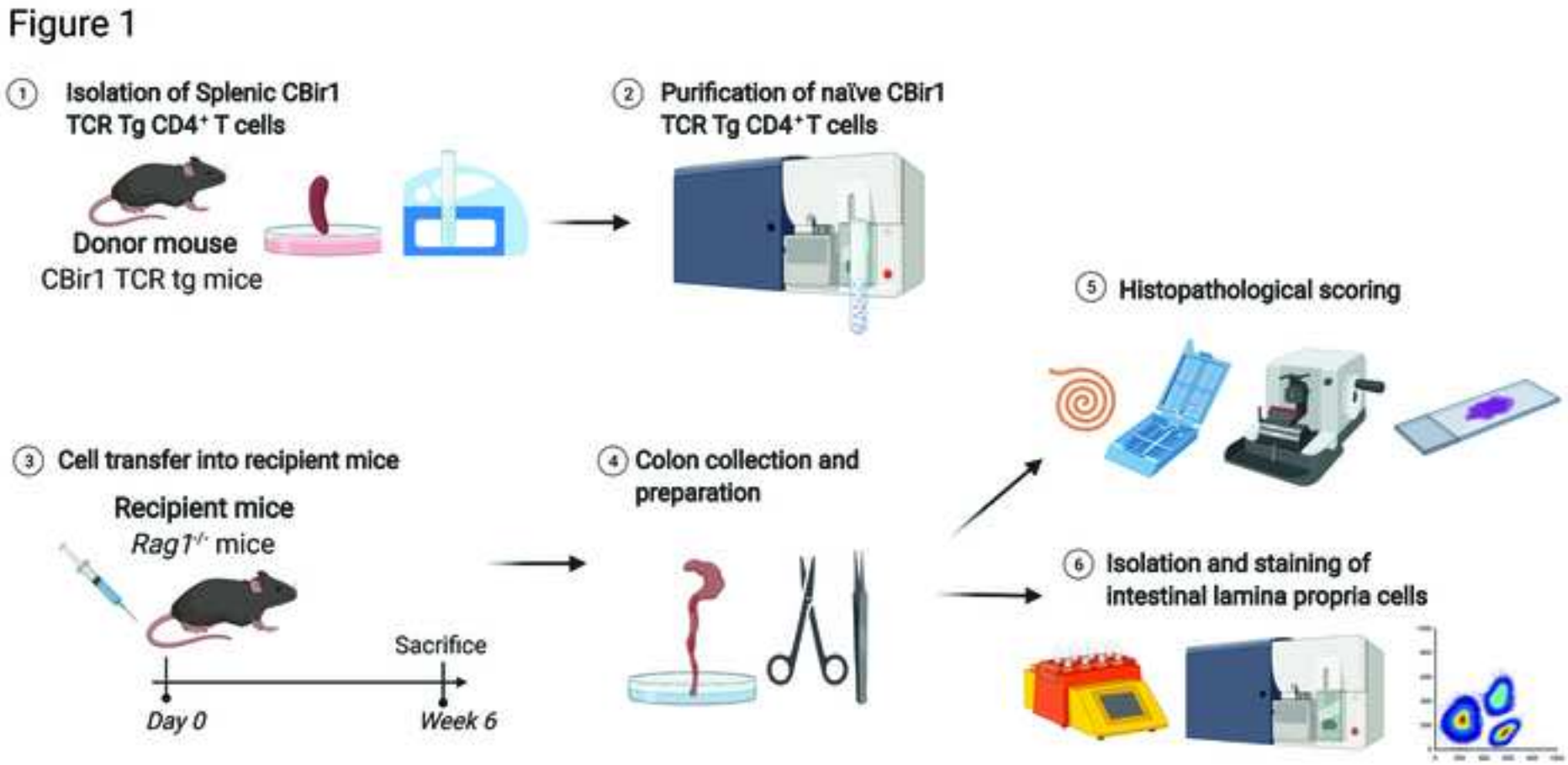


Figure 2

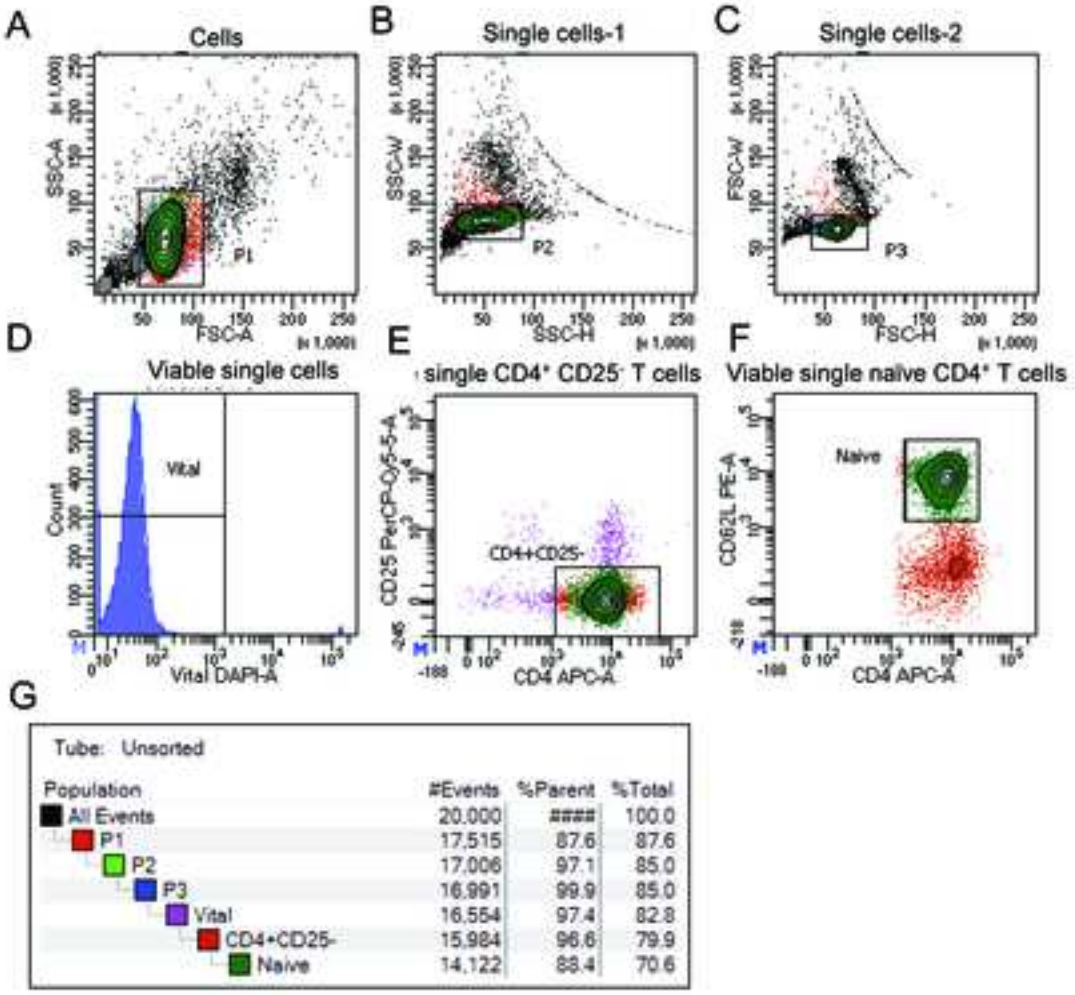
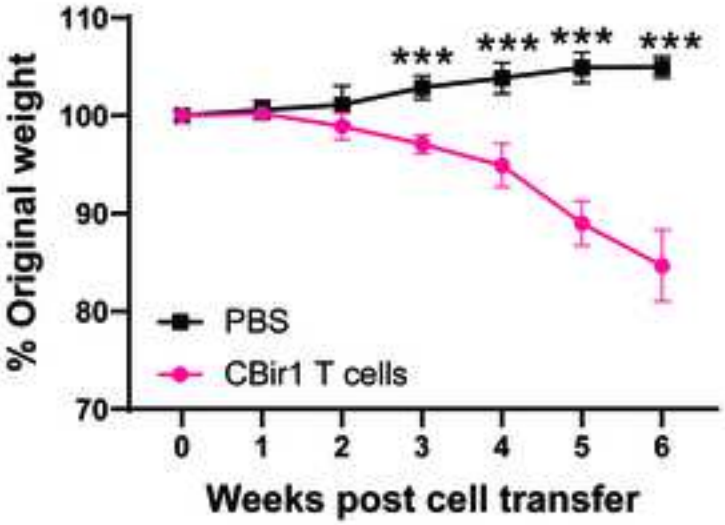


Figure 3



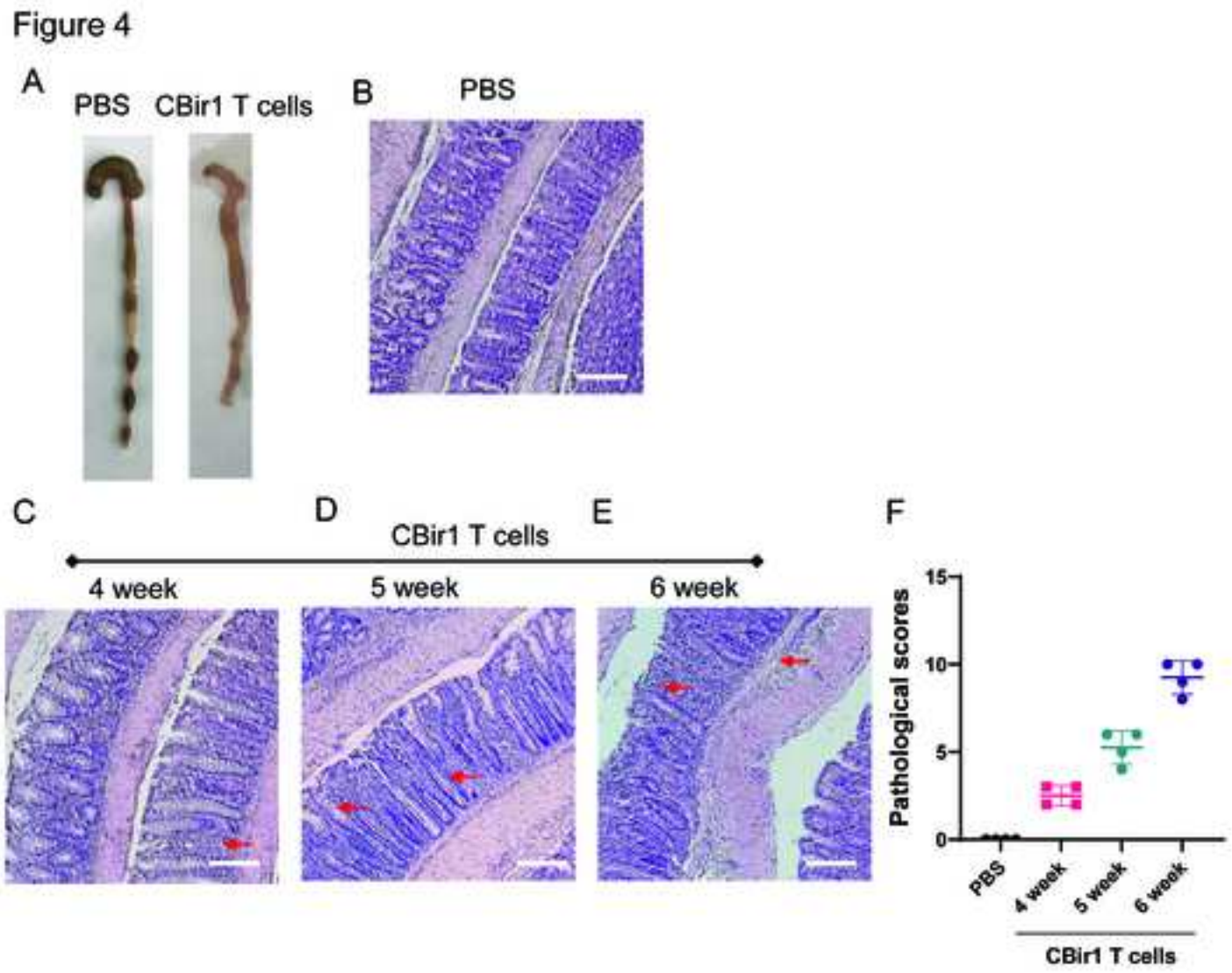
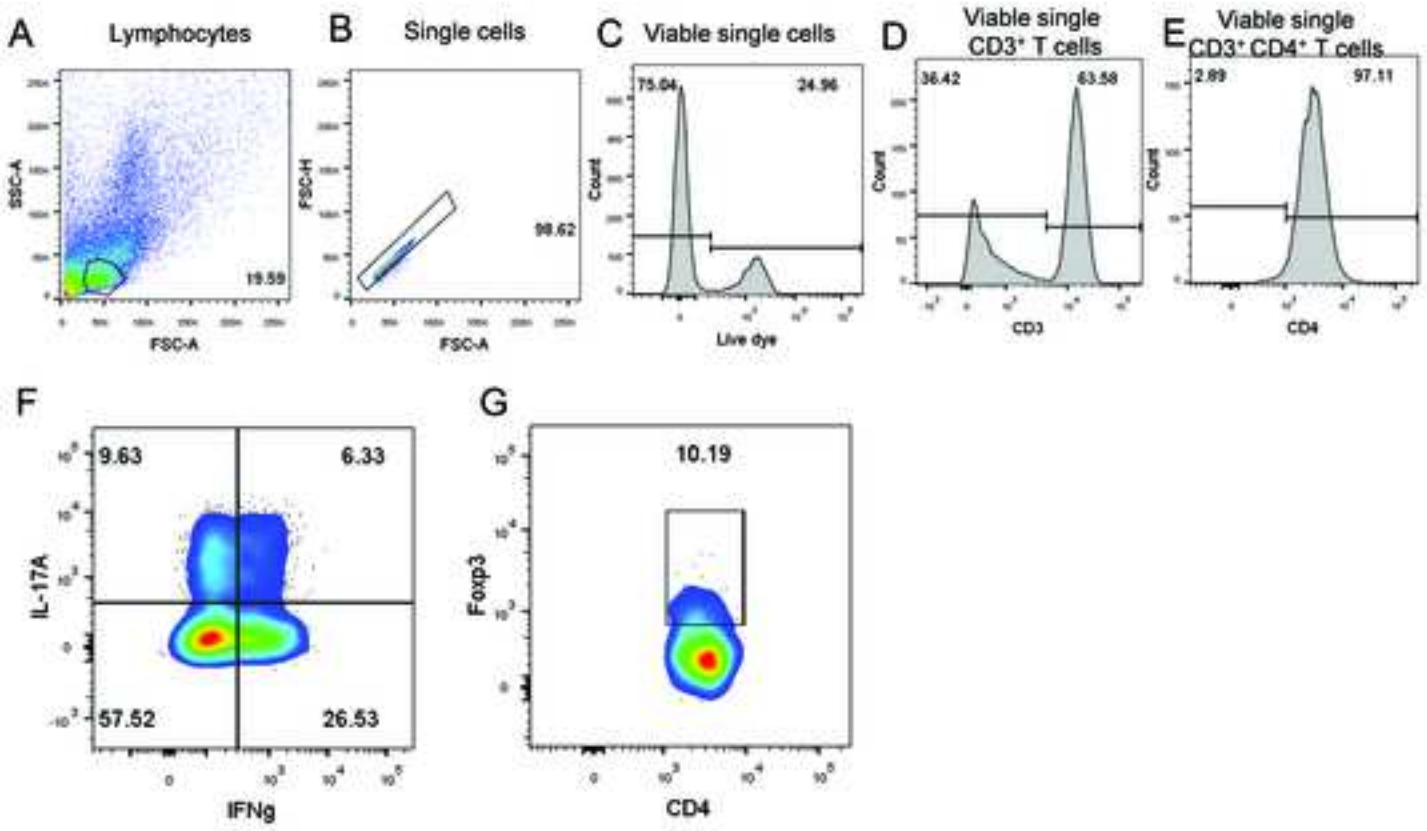


Figure 5



utmb Health

SCHOOL OF MEDICINE

DEPARTMENT OF MICROBIOLOGY/IMMUNOLOGY
Yingzi, Cong Ph. D
Professor

Nov 13, 2021

Nilanjana Saha, PhD
Review Editor
JoVE Journal

Dear Dr. Saha:

We are submitting the revised manuscript titled **"**Induction of intestinal inflammation by adoptive transfer of CBir1 TCR transgenic $CD4^+$ T cells to immunodeficient mice **"** (63293R1**)** for publication in *JoVE Journal***.** We have carefully read through the editorial review and thoroughly revised the manuscript in response to all the suggestions and comments. Thank you for your consideration of this revised manuscript

Sincerely yours,

Yingzi Cong, PhD
Samuel Baron Distinguished Professor
Professor of Microbiology, Immunology, and Pathology
Department of Microbiology and Immunology
University of Texas Medical Branch

utmb Health

SCHOOL OF MEDICINE

DEPARTMENT OF MICROBIOLOGY/IMMUNOLOGY
Yingzi, Cong Ph. D
Professor

Nov 11, 2021

Editors
JoVE Journal

Dear Editors:

We are submitting the revised manuscript titled **"Induction of intestinal inflammation by adoptive transfer of CBir1 TCR transgenic CD4$^+$ T cells to immunodeficient mice" (**63293R1**)** for publication in *JoVE Journal***.** We have carefully read through the reviews and thoroughly revised the manuscript in response to all the suggestions and comments of the reviewers and the editors. The changes in the manuscript are highlighted in red to assist the reviewers.

Reply to Editors

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*
Reply: Thanks for the suggestion. Done accordingly.

2. *Please reword the following lines to avoid previously published work: 20-21, 196-210.*
Reply: Done accordingly.

3. *Please avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.) in the manuscript text.*
Reply: Done accordingly.

4. *JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.*
For example: gentleMACS.
Reply: We have gone through the manuscript carefully and removed all the commercial language.

UNIVERSITY OF TEXAS MEDICAL BRANCH ROOM 4.142C MEDICAL RESEARCH BUILDING
301 UNIVERSITY BOULEVARD GALVESTON, TEXAS 77555-1019
(409) 772-4902 FAX (409) 772-5065
e-mail yicong@utmb.edu

5. *Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). Any text that cannot be written in the imperative tense (e.g., provide extraneous details, optional steps, or recommendations) may be added as a "NOTE."*
Reply: Done accordingly.

6. *Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.*
Reply: Thanks for the comments. After going through the manuscript thoroughly, we modified some steps and added more references.

7. *Please add more details to your protocol steps:*
Step 1/2: Please specify the age/gender/strain of the mouse used.
Reply: Done accordingly. Please see the revised manuscript.

Step 2.2: Please specify the incision size. Also please include the details of Penicillin-Streptomycin and RPMI in the Table of materials.
Reply: Done accordingly.

Step 2.4: Please mention the composition of Tris-NH4Cl Lysis Buffer.
Reply: Done accordingly. Additionally, we included the steps for the preparation of reagents. Please see the revised manuscript.

Step 2.6: Please include a citation for the trypan blue method for counting the cells.
Reply: Done accordingly.

Step 2.7/3.4/3.10/7.13: Please mention how was the aspiration done. Was a pipette used?
Reply: Done accordingly. Please see the revised manuscript.

Step 2.8: 5uL of the commercially available magnetic nanoparticles were directly added or any dilution was made?
Reply: Done accordingly. Please see the revised manuscript.

Step 6.2: Please mention the incision size.
Reply: Done accordingly.

Step 6.5: What are these cassette and automatic processor? Are these obtained commercially? Then please include the details in the Table of Materials.
Reply: They were Tissue Embedding/Processing Cassette and Tissue Automatic Processor, and can be obtained commercially, which was included in the Table of Materials in the revised version.
Step 7.12: How was the fat on the top layer removed? A pipette was used? Please specify.

UNIVERSITY OF TEXAS MEDICAL BRANCH ROOM 4.142C MEDICAL RESEARCH BUILDING
301 UNIVERSITY BOULEVARD GALVESTON, TEXAS 77555-1019
(409) 772-4902 FAX (409) 772-5065
e-mail yicong@utmb.edu

Reply: A pipette was used to remove the top layer. Please see the revised manuscript.

8. Please include one line space between the protocol steps and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
Reply: Done accordingly.

9. *Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next and also is in-line with the Title of the manuscript. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. However, the NOTEs and the steps including euthanasia/anesthesia cannot be filmed, so please do not highlight.*
Reply: Done accordingly.

10. Please modify the Result section to include all the observations and conclusions you can derive from the Figures. The Results section should focus on the effectiveness of your technique backed up with data.
Reply: I do appreciate the editors' comments. We have modified the Result section as suggested. Please see the revised manuscript.
11. As we are a methods journal, please ensure that the Discussion cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) significance with respect to existing methods
e) Any future applications of the technique
Reply: Done accordingly.

12. Figure 4B: Please include the x-axis description.
Reply: Done accordingly.

Reply to Reviewer #1:

1. The text needs to be significantly improved (e.g. line 20: "Inflammatory bowel diseases (IBD) are disorders of chronic intestinal inflammation"; line 34: "gastroenterology tract"; figure 1: "collon"); line 82: Mix the 10 µl of cell suspension (here the authors just take a 10 µL aliquot but not the complete 10 mL of the cell suspension), etc….. The authors need to keep the units harmonized (e.g. line 82: µl or µL). Why the authors use sometimes a hyphen between numbers and units of measurement (e.g. 50-mL)?
Reply: We do appreciate the reviewer's careful reading through the manuscript and apologize for the typos. We have carefully gone through and edited the revised manuscript.

UNIVERSITY OF TEXAS MEDICAL BRANCH ROOM 4.142C MEDICAL RESEARCH BUILDING
301 UNIVERSITY BOULEVARD GALVESTON, TEXAS 77555-1019
(409) 772-4902 FAX (409) 772-5065
e-mail yicong@utmb.edu

Additionally, we used 10 µL of cell suspension for counting the cells, and the remaining cell suspension was spun down for the following steps. To avoid the misunderstanding, I re-worded those steps. Please see the revised manuscript.
As suggested, we changed all the 'µl' to 'µL'. Besides, we used a hyphen between numbers and units only for the tubes/ strainers.

2. The authors should give the characteristics of the immune-deficient recipient mice to be used in the model including Rag2-/- or TCRβ/δ-/-. The authors isolate the naïve CD4+ T lymphocytes by using the markers CD4, CD25 and CD62L, I believe that to justify the use of these markers would be appreciated by researchers who are not immunologists.
Reply: We do appreciate the reviewer's suggestion. Done accordingly.

3. Step 4.3: line 126: the size of the needle for insulin syringe (29G?) and the site of injection (injection into tail vein as indicated in the discussion section?) should be indicated. It may also be indicated that the injection can be performed under a light isoflurane anesthesia.
Reply: We do appreciate the reviewer's comments. We used 27G needle for insulin syringe (see Cat# in the Table of Materials), and injected the cells into tail veins, which was included in the revised manuscript. Per our ARC protocol, we did not use anesthesia.

4.The Step 5 is too succinctly described: the clinical disease index should be described in details with corresponding colitis severity scoring.
Reply: We do appreciate the reviewer's suggestion. However, we do not have the clinical sign scoring. We monitor the clinical signs because these signs indicate the model is successful, which was described in the Result section. Besides, weight loss > 20% of the original weight is also a sign of euthanasia, which was described in the Step 7.1.

5.Step 6.6. It is not necessary to describe H&E staining but a reference would be useful.
Reply: Done accordingly.

6. Step 6.7. Examples of histological scores ranging from healthy (not transferred mice) to very severe colitis will improve the figure 4. The authors should, at least describe the histological tissue damage depicted in the figure 4A (right panel).
Reply: We do appreciate the reviewer's suggestion. Please see the revised Figure 4.

7. Step 7.6 what is the program 37C-M-LPDK-1? (characteristics?)
Reply: This is the standard program in the Dissociator, which was pre-set by Miltenyi Biotec. Therefore, it is unfortunately that we do not know the exact procedures under this program, but it is used for stirring the samples and keeping the samples at 37 °C.

8. Step 7.9 and 7.10 are confusing. All the supernatants are aspirate and then discard?
Reply: We do apologize for the confusion. The supernatants were just discarded. Please see the revised manuscript.

9. Step 7.12. Centrifugation without acceleration?
Reply: We have re-worded this sentence. Please see the revised manuscript.

UNIVERSITY OF TEXAS MEDICAL BRANCH ROOM 4.142C MEDICAL RESEARCH BUILDING
301 UNIVERSITY BOULEVARD GALVESTON, TEXAS 77555-1019
(409) 772-4902 FAX (409) 772-5065
e-mail yicong@utmb.edu

10. Step 7.19.2. It may be mentioned that the reagents used are toxic and their manipulation needs precautions and safety measures.
Reply: Done accordingly.

11. Figure 5. The origin of FoxP3+Treg found in colitis mice should be discussed.
Reply: The Foxp3+ Treg cells were differentiated from naïve T cells, which was discussed in the revised manuscript.

Reply to Reviewer #2:

1. It will be helpful if the authors add representative images of intestine (or colon) to show the gross changes in mice with colitis. It will give the readers a general idea of how the colitic colons look like if the model is successfully established.
Reply: We do appreciate the reviewer's suggestion. Done accordingly. Please see the revised Figure 4.

Reply to Reviewer #3:

1. The key mouse strain required (CBir1) is not commercially available. The protocol provides no information on where the authors obtained it from or how readers could obtain it in the future. This seriously limits the usefulness of the protocol.
Reply: The CBir1 Tg mice were provided by Dr. Charles Elson at the University of Alabama at Birmingham. The information was included in the revised manuscript.

2. More information is needed to justify the use of CBir over non-TCR transgenic naïve T cells (which will also potently induce colitis). The work of Chiaranunt et al (PMID 29777027 - this should be cited), shows that the majority of CBir1 T cells that expand in a lymphopenic host do not recognize Fla antigens due to other rearranged endogenous alpha chains. This may restrict the utility of this model in assessing Fla antigen-specific responses - the main justification for using CBir1 cells provided in the discussion.
As accumulating evidence indicates a crucial role of gut bacterial antigen-specific reactive T cells in the pathogenesis of IBD, using T cells specific for a defined gut bacterial antigen will provide insights into how gut bacterial antigen induce T cell responses to induce colitis. Interestingly, the recent work of *Chiaranunt* et al. indicated that T cell specificity to the microbiota alone might not be sufficient to induce T cell activation and colitis as while WT CBir1 T cells induced colitis in Rag KO recipients, $Rag^{-/-}$ CBir1 T cells did not induce colitis in their animal facility, suggesting that gut T cells respond to specific gut bacterial antigen may require other interrelated commensal bacteria, for example, *Helicobacter* spp, to function as an adjuvant. This information was included, and Chiaranunt et al.'s paper was cited in the revised manuscript.

3.It would be helpful to provide some estimates of the number of cells obtained at each stage of

UNIVERSITY OF TEXAS MEDICAL BRANCH ROOM 4.142C MEDICAL RESEARCH BUILDING
301 UNIVERSITY BOULEVARD GALVESTON, TEXAS 77555-1019
(409) 772-4902 FAX (409) 772-5065
e-mail yicong@utmb.edu

the purification and therefore an estimate of the number of donor mice one would need for a given number of recipients.
Reply: We do appreciate the reviewer's suggestion. Done accordingly.

4. Line 61. It is important to stress the importance of sex matching between donor and recipient, not just within groups.
Reply: We do appreciate the reviewer's comment. In this model, it is not necessary for sex matching between donor and recipient, which was discussed in the revised Discussion section.

5. Line 65. This sentence does not make sense to me.
Reply: Thanks for the comment. We have re-worded this sentence.

6. Line 114. Please provide more details of your recommended sorting setup. Nozzle size, pressure, event rates etc. This will vary between individual machines, but it is useful information to have to give readers a general idea of what you recommend.
Reply: We do appreciate the reviewer's suggestion. Done accordingly.

7. Line 147. Please provide a reference for this scoring system. If it is a system you have developed yourselves, please provide representative images to demonstrate the various scoring levels.
Reply: We do appreciate the reviewer's suggestion. The reference was added, and more representative images were also included in the revised manuscript.

8. Line 173. Please provide more details about how the Percoll is prepared (what is it diluted in?)
Reply: We do appreciate the reviewer's suggestion. Done accordingly. Besides, we included the preparation of all the reagents in the revised protocol.

9. Line 183. This concentration of PMA is very high - is it correct?
Reply: Yes, we used PMA at the concentration of 50 ng/mL.

10. Line 184. Please provide a concentration of BFA used.
Reply: We do appreciate the reviewer's comment. Done accordingly.

Reply to Reviewer #4:

1. The CBir1 TCR Tg mice are not commercially available (Jax, Taconic). Therefore, it would be helpful to include the information on how we can obtain the line (need MTA with who? Etc.).
Reply: See response to Reviewer #3, question #1. The CBir1 Tg mice were provided by Dr. Charles Elson at the University of Alabama at Birmingham. The information was included in the revised manuscript.

2. Please indicate what better controls will be for FACS analysis. In Rag KO host without transferring cells, no T cells can be found, and it is not appropriate control. Please argue this.

UNIVERSITY OF TEXAS MEDICAL BRANCH ROOM 4.142C MEDICAL RESEARCH BUILDING
301 UNIVERSITY BOULEVARD GALVESTON, TEXAS 77555-1019
(409) 772-4902 FAX (409) 772-5065
e-mail yicong@utmb.edu

Reply: We do appreciate the reviewer's comment. The purpose of FACS analysis is to define T cell phenotypes in colitic mice induced by transferred CBir1 T cells. As there are no Rag KO mice do not develop colitis after receiving CBir1 T cells, it is impossible to have healthy Rag KO mice with transferred T cells to serve as controls. Thus, it is appropriate to use Rag KO mice without transferred T cells to serve as controls.

3. The negative gate of CD25 is very important to remove regulatory T cells. However, it is a little bit unclear how the other antibodies must be chosen. Some investigators used different antibodies to sort Naïve T cells.
A. CD44+ CD62L- CD4+ TCRb+ CD25-
B. CD45RB High CD4+ CD25-
It would be really useful if the authors included this information.
Reply: We do appreciate the reviewer's comment. $CD4^+$ T cells belong to TCRαβ T cells, so some researchers used the combination of TCRβ and CD4 as the markers of $CD4^+$ T cells. CD62L is highly expressed in naïve T cells, and CD44 and CD25 are the activation markers of T cells. CD45RA and CD45RB are also a marker of naïve T cells.
In this study, we first used anti-CD4 magnetic beads to isolate CD4+ T cells from spleens. Then we used the combination of anti-CD4, anti-CD62L, and anti-CD25 antibodies for isolation of naïve $CD4^+$ T cells. Researchers could use other markers for sorting the naïve $CD4^+$ T cells. Additionally, $CD45RB^{hi}$ $CD25^-$ $CD4^+$ T cells are commonly used as naïve T cells of WT non-TCR transgenic T cells.

4. The author used just spleen, not LNs. It would be very appreciated if the authors could mention it is ideal to use only the spleen, or it can be possible to include other LNs, including mLNs (or should not include mLNs).
Reply: We do appreciate the reviewer's comment. MLNs could also be used for the isolation of naïve $CD4^+$ T cells. However, the yield of naïve $CD4^+$ T cells in MLNs and other LNs is much lower than in spleens. Therefore, we recommend using splenic T cells. This point was also included in the Discussion section.

5. Is there any dose dependency for the number of transferred CBir1 TCR Tg Naïve CD4 T cells?
Reply: We do appreciate the reviewer's comment. The time to develop colitis varies when the different number of CBir1 TCR Tg Naïve CD4 T cells are transferred. As expected, fewer cells require a longer time for induction of colitis, and a higher number of cells require a shorterer time for induction of colitis. We recommend using 1×10^6 cells per recipient mouse since the recipient mice demonstrate clinical signs of colitis around 2-3 weeks post cell transfer and develop relatively severe colitis around 6 weeks post cell transfer.

6. It would be appreciated if the authors could provide any examples of pathology scoring (described in step 6.7) in Figure 4. The authors should use asterisks or arrowheads to indicate the area of inflammation and the type of pathology (parameters such as abnormal crypt etc.), at least.
Reply: We do appreciate the reviewer's suggestion. Done accordingly. Please see the revised Figure 4.

UNIVERSITY OF TEXAS MEDICAL BRANCH ROOM 4.142C MEDICAL RESEARCH BUILDING
301 UNIVERSITY BOULEVARD GALVESTON, TEXAS 77555-1019
(409) 772-4902 FAX (409) 772-5065
e-mail yicong@utmb.edu

7. Some use IV or RO. It would be very appreciated if the authors could raise the differences between IV and RO routes in this model or general WT naïve T cell transfer model.
Reply: We do appreciate the reviewer's comment. Both IV and RO routes work equally well. Some researchers also use IP route. In this protocol, we used intravenous injection (IV) into tail vein. Compared with an intraperitoneal injection (IP), IV transfer of cells induce more consistent colitis in either the CD45RBhi T cell transfer model or CBir1 Tg naïve T cell transfer model. This point was also included in the revised Discussion section.

8. No inflammation in the small intestine or cecum?
Reply: We do appreciate the reviewer's comment. There is no inflammation in the small intestine, but the cecum has inflammation. This point was also included in the Result section.

9. Are there any phenotypic differences between males and females?
Reply: We do appreciate the reviewer's comments. We did not see a significant difference between recipient males and females, which was also included in the revised manuscript.

10. What are the phenotypic differences between regular WT naïve T cell transfer colitis and CBir1 TCR Naïve CD4 T cell transfer colitis?
Reply: There are no reports to carefully compare the phenotypic differences between regular WT naïve T cell transfer colitis and CBir1 TCR Naïve CD4 T cell transfer colitis.

11. Is there any information about the case of CBir1 TCR Naïve CD8 T cell transfer into Rag KO hosts?
Reply: We do appreciate the reviewer's comment. Unfortunately, there is no information about the case of CBir1 TCR Naïve CD8 T cell transfer into Rag KO hosts.

12. It has been mentioned that naïve T cells from male and female donors can be transferred to male Rag KO recipients. If female RAG−/− recipients are to be used, then only female WT donors may be used (PMID: 19033538). Can the author agree with this? If so, it is worth indicating this. If not, please mention the reasons.
Reply: We do appreciate the reviewer's comments. In our animal facility, we did not see this phenomenon. The recipient mice could be either females or males, and T cells from male donors can also induce colitis in female recipients. This point was included in Result Section and Discussion Section.

13. Please have a discussion about this paper (PMID: 29777027).
Reply: See response to Reviewer #3, question #2. The CBir1 Tg T cells are reactive to a defined gut bacterial antigen CBir1 flagellin, whereas WT non-TCR transgenic naïve T cells may respond to multiple gut bacterial antigens. As accumulating evidence indicates a crucial role of gut bacterial antigen-specific reactive T cells in the pathogenesis of IBD, using T cells specific for a defined gut bacterial antigen will provide insights into how gut bacterial antigen induce T cell responses to induce colitis. Interestingly, the recent work of Chiaranunt et al indicated that T cell specificity to the microbiota alone may not be sufficient to induce T cell activation and colitis as while WT CBir1 T cells induced colitis in Rag KO recipients $Rag^{-/-}$ CBir1 T cells did not induce colitis in their animal facility, suggesting that gut T cells respond to specific gut

UNIVERSITY OF TEXAS MEDICAL BRANCH ROOM 4.142C MEDICAL RESEARCH BUILDING
301 UNIVERSITY BOULEVARD GALVESTON, TEXAS 77555-1019
(409) 772-4902 FAX (409) 772-5065
e-mail yicong@utmb.edu

bacterial antigen may require other interrelated commensal bacteria, for example, *Helicobacter* spp, to function as adjuvant. This information was included in the revised manuscript.

Reply to Reviewer #5:

1. This protocol is broadly use in the experimental IBD field and is proposed by the authors to be commensal specific. Using WT naive CD4 T cells is also commensal specific as seen by experiments in which the microbiota is depleted with antibiotics.
Reply: We do appreciate the reviewer's comments. We agree that CD45RB^{hi} CD4^{+} T cell are also responsive to commensal microbiota. However, intestinal microbial antigens recognized d by CD45RB^{hi} CD4^{+} T cell are not specific and not well-identified. Instead, CBir1 TCR Tg naïve T cells are specific CBir1 flagellin and induce chronic colitis after adoptive transfer into immune-deficient *Rag*$^{-/-}$ mice.

2. In addition, the authors do not discuss the potential caveat in using polyclonal CBir transgenic naive CD4 T cells (from a WT-CBir mice) in comparison with monoclocal Cbir obtained from RAG-/-Cbir mice (see doi: 10.4049/jimmunol.1800236).
Reply: **Reply**: See response to Reviewer #3, question #2. The CBir 1 Tg T cells are reactive to a defined gut bacterial antigen CBir1 flagellin, whereas WT non-TCR transgenic naïve T cells may respond to multiple gut bacterial antigens. As accumulating evidence indicates a crucial role of gut bacterial antigen-specific reactive T cells in the pathogenesis of IBD, using T cells specific for a defined gut bacterial antigen will provide insights into how gut bacterial antigen induce T cell responses to induce colitis. Interestingly, the recent work of Chiaranunt et al indicated that T cell specificity to the microbiota alone may not be sufficient to induce T cell activation and colitis as while WT CBir1 T cells induced colitis in Rag KO recipients Rag$^{-/-}$ CBir1 T cells did not induce colitis in their animal facility, suggesting that gut T cells respond to specific gut bacterial antigen may require other interrelated commensal bacteria, for example, *Helicobacter* spp, to function as adjuvant. This information was included in the revised manuscript.

3. Also, it is not clear why the authors use 1 million cells instead of the 400,000 ususally used in RBhi colitis models. This rise the ethical issue that goes against the 3R pronciple of reducing and refinement.
Reply: We do appreciate the reviewer's comments. First, the cell number is used differently among different animal facilities, even CD45Rbhi T cell transfer model, as microbiota and other environmental factors could not be exactly the same in different facilities. Second, these two colitis models are not identical. Therefore, the capacity of inducing colitis is not the same. We tried different cell numbers and found that it took different times for inducing colitis when the transfer of the different numbers of CBir1 TCR Tg Naïve CD4 T cells. As expected, fewer cells require a longer time for induction of colitis and a higher number of cells require a shorter time for induction of colitis. We recommend using 1×10^6 cells per recipient mouse since the recipient mice demonstrated clinical signs of colitis around 2-3 weeks post cell transfer and developed relatively severe colitis around 6 weeks post cell transfer.

UNIVERSITY OF TEXAS MEDICAL BRANCH ROOM 4.142C MEDICAL RESEARCH BUILDING
301 UNIVERSITY BOULEVARD GALVESTON, TEXAS 77555-1019
(409) 772-4902 FAX (409) 772-5065
e-mail yicong@utmb.edu

4. How does the CD62L+ CD4 T cell isolation compare to the classical CD45RBhi protocol? Have the authors checked the CD45RB levels of their purified populations?
Reply: We do appreciate the reviewer's comment. CD62L is highly expressed in naïve T cells, and CD44 and CD25 are the activation markers of T cells. CD45RA and CD45RB are also a marker of naïve T cells. $CD45RB^{hi}$ $CD4^{+}$ T cells are subtypes of naïve T cells, which are responsive to the intestinal microbiota.
Therefore, all the CBir1 TCR Tg naïve $CD4^{+}$ T cells express CD45RB, including $CD45RB^{medium}$ and $CD45RB^{hi}$.

5. Could differences in the purity of naïve T cells explain the need for one million cells injected per mouse in this model compared to a published CD45RBhi model in which only 400.000 cells are sufficient to induce colitis (Read and Powrie, Currrent Protocols in Immunology 1999)? Have you tried injecting less and do you see the same? We have an ethical concern on the number of mice needed to perform this experiment.
Reply: We do appreciate the reviewer's comments. First, the cell number is used differently among different animal facilities, even CD45Rbhi T cell transfer model, as microbiota and other environmental factors could not be exactly the same in different facilities. Second, these two colitis models are not identical. Therefore, the capacity of inducing colitis is not the same. We tried different cell numbers and found that it took different times for inducing colitis when the transfer of different numbers of CBir1 TCR Tg Naïve CD4 T cells. As expected, fewer cells require a longer time for induction of colitis and a higher number of cells require a shorter time for induction of colitis. We recommend using 1×10^6 cells per recipient mouse since the recipient mice demonstrated clinical signs of colitis around 2-3 weeks post cell transfer and developed relatively severe colitis around 6 weeks post cell transfer.

6. In step 6, is histological scoring done on the distal 1/3 of the colon only or do the authors observe differences between proximal and distal colon?
Reply: We do appreciate the reviewer's comment and apologize for the confusion we made. We cut 1/3 of the colon longitudinally, which includes the proximal and distal colon. The inflammation exits both in the proximal and distal colon.

7. Rag-/- mice have an expanded ILC fraction in their intestines which is a confounding factor when analyzing the phenotype of the injected CD4 T cells. We recommend adding a CD3 antibody to rule out that ILCs may be contributing to the phenotype seen in Figure 5. Could the authors please show the pre-gating for the flow cytometry plots shown in Figure 5?
Reply: We do appreciate the reviewer's suggestion. Done accordingly. Please see the revised Figure 5.

8. *Importantly, our lab has confirmed the finding of a previous study (Chiaranunt, JI 2018) that specificity for the CBir1 antigen is insufficient to initiate colitis in the CD45RBhi model. The authors claim that "The major advantage of this colitis model is that it is great to investigate the role of microbiota-specific antigen reactive T cells in regulating the pathogenesis of intestinal inflammation." How do the authors explain that this model is superior to the classical*

CD45RBhi model where transferred cells are polyclonal in light of the Chiaranunt study's findings?
Reply: We do appreciate the reviewer's comments. We do not state that CBir1 T cell transfer model is superior to the classical CD45RBhi transfer model. As accumulating evidence indicates a crucial role of gut bacterial antigen-specific reactive T cells in the pathogenesis of IBD, using T cells specific for a defined gut bacterial antigen will provide insights into how gut bacterial antigen induce T cell responses to induce colitis. The elegant work of Chiaranunt et al. demonstrated the complexity of T cell response to gut bacterial antigens in real-world which requires multiple levels of interactions between host and gut microbiota and interactions between gut bacterial ecology. As WT CBir1 T cells induce colitis, it provides an ideal model to study how different gut bacteria interact to regulate T cell responses to specific antigens to induce colitis.

9. Write that CO2 is a form of anesthesia is just wrong.
Reply: We do appreciate the reviewer's comment and apologize for the typo. We have corrected it in the revised manuscript.

10. As a minor point, please check typos (i.e. line 67, 86, 133).
Reply: We do appreciate the reviewer's careful reading through the manuscript. We have changed it in the revised manuscript.

11. The concentrations of components used in the lysis buffer in line 77 should be given.
Reply: We do appreciate the reviewer's suggestion. Done accordingly. Besides, we included the preparation of all the reagents in the revised protocol.

We thank the editors and the reviewers for their careful reading of the manuscript and especially for their helpful comments. With the inclusion of the responses provided above, we believe that we have responded in full to the comments of the three reviewers and editors. We trust that this revised manuscript will now be suitable for publication in *JoVE Journal*. Thank you for your consideration of this revised manuscript

Sincerely yours,

Yingzi Cong, PhD
Samuel Baron Distinguished Professor
Professor of Microbiology, Immunology, and Pathology
Department of Microbiology and Immunology
University of Texas Medical Branch