

Mouse Model of Reversible Intestinal Inflammation

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[Abstract] Current therapies to treat inflammatory bowel disease by dampening excessive inflammatory immune responses have had limited success (Reinisch *et al.*, 2011; Rutgeerts *et al.*, 2005; Sandborn *et al.*, 2012). To develop new therapeutic interventions, there is a need for better understanding of the mechanisms that are operative during mucosal healing (Pineton de Chambrun *et al.*, 2010). To this end, a reversible model of colitis was developed in which colitis induced by adoptive transfer of naïve CD4⁺ CD45RB^{hi} T cells in lymphopenic mice can be reversed through depletion of colitogenic CD4⁺ T cells (Brasseit *et al.*, 2016).

Keywords: Colitis, Remission, Relapsing disease, Mucosal healing

[Background] Our understanding of the pathogenesis of inflammatory bowel disease (IBD), which is a chronic inflammatory disorder of the intestine, has been greatly improved with the development of animal models aiming to recapitulate human disease (Khanna *et al.*, 2014). Despite the identification of a wide array of immunological targets, current therapies have had limited success in treating IBD and limited knowledge is available about the mechanisms that are induced in the establishment of long-term remission and the associated mucosal healing (D'Haens *et al.*, 2014). A major limitation, so far, has been the lack of animal models in which remission can be reproducibly induced in animals with established disease. In models of infection induced intestinal inflammation, pro-inflammatory and anti-inflammatory mechanisms can be operative simultaneously, implying that dissecting the role of different immune pathways during resolution of inflammation may be a challenge (Endt *et al.*, 2010; Sonnenberg *et al.*, 2011). In dextran sodium sulfate (DSS) induced colitis, DSS can be easily administered in the drinking water to either induce acute or chronic intestinal inflammation and this is followed by treatment with normal drinking water to study resolution of colitis. However, the kinetics and severity of disease are highly dependent on numerous factors including differences in the dose of DSS used and critically on the amount of DSS consumed which is virtually impossible to normalize between different animals of the same cage (Chassaing *et al.*, 2014; Perše and Cerar, 2012). Using the T cell transfer mediated colitis model, it was elegantly shown that intestinal inflammation can be reversed by the adoptive transfer of CD45RB^{lo} regulatory T cells (Treg) in colitic animals, resulting in remission induction within 10-14 weeks post Treg transfer (Mottet *et al.*, 2003). The kinetics of remission induction however varies depending on the expansion of transferred Treg and it can be difficult to synchronize the onset of remission between animals of same experimental group. To overcome the unpredictable timing and extent of remission induction, we developed a new mouse model of reversible intestinal inflammation in

which intestinal inflammation (induced by the adoptive transfer of naïve CD45RB^{hi} T cells in lymphopenic animals) can be reversed by depletion of colitogenic CD4⁺ T cells in mice with established disease, resulting in reproducible induction of remission from colitis (Brasseit *et al.*, 2016).

Materials and Reagents

1. 50 ml tube (SARSTEDT, catalog number: 62.547.254)
2. 5 ml Falcon polystyrene (PS) round bottom tube (Corning, Falcon®, catalog number: 352058)
3. 100 µm nylon cell strainer (Corning, catalog number: 431752)
4. Glass slide Superfrost (Biosystems, catalog number: 85-0551-00)
5. 6-10 week old congenic CD90.1 or CD45.1 mice as donors (in-house bred mice on C57BL/6 background, initially purchased from THE JACKSON LABORATORY, USA)

Notes:

- a. *Congenic mice CD90.1 are used as donors to easily distinguish between donor (CD90.1) and recipient cells (CD90.2) but C57BL/6 mice can also be used as donors.*
- b. *It is possible to use donor mice older than 10 weeks old but the frequency of naïve CD4⁺ CD45RB^{hi} T cells is expected to decrease with age.*
6. 10-16 week old *Helicobacter* positive C57BL/6 Rag2^{-/-} or Rag1^{-/-} mice (initially purchased from THE JACKSON LABORATORY, USA) housed under specific pathogen-free (SPF) conditions as recipients with a minimum of 20 g body weight

Notes:

- a. *Helicobacter negative SPF Rag2^{-/-} (or Rag1^{-/-}) mice may also be used as recipients. However, the kinetics of colitis induction is delayed and the variation in disease activity may greatly vary at a given time post CD4 T cell-mediated colitis induction in those Helicobacter negative mice.*
- b. *Positivity for the presence of Helicobacter can be assessed by PCR using genomic DNA extracted from fecal pellets as previously described by Brasseit *et al.*, 2016.*
- c. *We have not observed any differences in the kinetics of colitis induction between male and female recipients.*
7. 0.5 M EDTA (Sigma-Aldrich, catalog number: 27285), in sodium form
8. EasySep™ Mouse Streptavidin RapidSpheres™ Isolation Kit (STEMCELL Technologies, catalog number: 19860)
9. InVivoMAb anti-mouse CD4 (Clone GK1.5) (BioxCel, catalog number: BE0003-1)
10. Anti-mouse antibodies

Antibodies	Company	Catalog number	Clone	Dilution
B220 Biotin	BioLegend	103203	RA3-6B2	1:800
CDαBiotin	BioLegend	100703	53-6.7	1:200
CD45RB FITC	Affymetrix	11-0455-85	C363-16A	1:800
CD25 PE	BioLegend	102008	PC61	1:800
CD4 APC-Cy7	BioLegend	100526	RM4-5	1:800

11. Horse serum (HS) (Sigma-Aldrich, catalog number: H1270)
12. Trypan blue solution (Sigma-Aldrich, catalog number: T8154)
13. 4% buffered formalin (EMD Millipore, catalog number: 100496)
14. Ethanol (EMD Millipore, catalog number: 100983)
15. Xylene (VWR, catalog number: 28975)
16. Paraffin (Engelbrecht, catalog number: 17932)
17. Mayer's hemalum (Hematoxylin) solution (EMD Millipore, catalog number: 109249)
18. Xylene based mounting medium Eukitt® (Sigma-Aldrich, catalog number: 03989)
19. Sodium chloride (NaCl) (EMD Millipore, catalog number: 106406)
20. di-Sodium hydrogen phosphate dehydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) (EMD Millipore, catalog number: 106580)
21. di-Potassium hydrogen phosphate trihydrate ($\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$) (EMD Millipore, catalog number: 105099)
22. Ammonium chloride (NH_4Cl) (Sigma-Aldrich, catalog number: 31107)
23. Potassium hydrogen carbonate (KHCO_3) (EMD Millipore, catalog number: 104854)
24. Eosin-Phloxine solution (VWR, catalog numbers: 341973R and 10047229, respectively)
25. Glacial acetic acid (EMD Millipore, catalog number: 100063)
26. Hydrochloric acid (EMD Millipore, catalog number: 100317)
27. Phosphate-buffered saline (PBS) (see Recipes)
28. ACK red blood lysis buffer (see Recipes)
29. Eosin-Phloxine solution (see Recipes)
30. 0.5% HCl-ethanol (see Recipes)

Equipment

1. Centrifuge
2. Neubauer chamber (Hemocytometer)
3. EasySep™ magnet (STEMCELL Technologies, catalog number: 18000)
4. BD FACS ARIA III (BD, model: BD FACS ARIA III)
5. Tissue-Tek VIP 6 tissue processor (SAKURA, model: Tissue-Tek VIP 6 Vacuum Infiltration Processor)
6. Tissue-Tek® uni-cassette® (SAKURA, catalog number: 4172)
7. Heating block
8. Dissection kit (forceps and scissors)
9. Cold plate (-10 °C)
10. Microtome (Leica Biosystems, Wetzlar, Germany)
11. Water bath
12. Pipette
13. CO₂ chamber

Note: CO₂ is used as an approved and recommended euthanasia method in accordance with the Swiss Federal and Cantonal animal experimental regulations.

Software

1. Graphpad Prism 6 (La Jolla, CA; <https://www.graphpad.com/scientific-software/prism/>)

Procedure

A. Selection of naïve CD4⁺ T cells

1. On day 0, isolate spleens from donor CD90.1 mice. Use 1 spleen per 2 recipient *Rag1*^{-/-} mice.
2. Disaggregate spleens on a 100 µm nylon filter and rinse the filter with PBS/2% horse serum (HS) to obtain a single cell suspension.
3. Spin down cell suspension at 370 x g for 5 min.
4. Lyse the red blood cells by resuspending the cell pellet in 3 ml of ACK lysis buffer for 3 min.
5. Wash cells with PBS/2%HS and resuspend the cell pellet in 1 ml of PBS/2% HS containing 5 mM EDTA.

Note: The initial volume for cell resuspension is not critically important since the cells have to be counted and adjusted to 1 x 10⁸ cells/ml, as indicated in the following step.

6. Count the cell numbers using a hemocytometer and adjust the cell concentration to 1 x 10⁸ cells/ml.
7. Add 50 µl/ml of the provided rat serum (from EasySep™ Mouse Streptavidin RapidSpheres™ Isolation Kit) and incubate with biotinylated antibodies (anti-mouse B220 and CD8α). Mix the cells carefully by pipetting.
8. Leave the tube containing the cell suspension on ice for 15 min.

Note: Cells have to be mixed carefully by pipetting following addition of anti-mouse antibodies. The tube containing the cell resuspension can be left on ice without further mixing/inverting/shaking.

9. Vortex the streptavidin RapidSpheres™ for 30 sec and add 85 µl/ml of RapidSpheres™ to the cell suspension.

Note: No initial wash step of the RapidSpheres™ is required but vortexing the RapidSpheres™ is critical since they tend to settle at the bottom of the tube.

10. Mix carefully by pipetting up and down and incubate at room temperature for 2.5 min.
11. Bring the cell suspension to a total volume of 2.5 ml using PBS/2% HS containing 5 mM EDTA and transfer to a 5 ml PS tube.
12. Place the 5 ml PS tube containing the cell suspension into the EasySep™ magnet for 2.5 min at room temperature.

13. In one continuous motion, invert the magnet and carefully pour off the cell fraction (streptavidin RapidSpheres™ unbound fraction) into a new 5 ml tube, leaving the magnet and tube in an inverted position for 2-3 sec to ensure optimal transfer of the cell suspension.

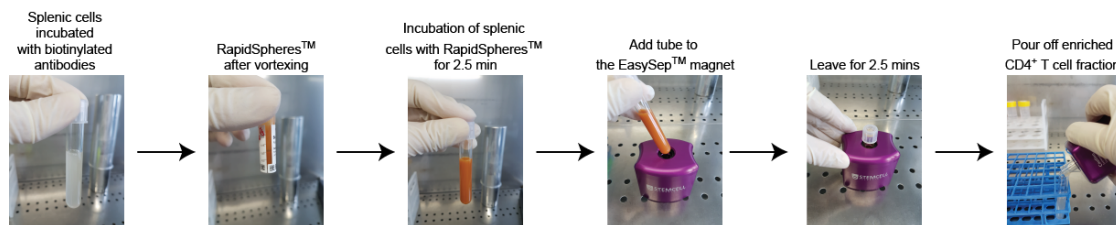


Figure 1. T cell enrichment method using splenocytes of donor mice

14. Wash cells in PBS/2% HS at 370 x g for 5 min.
15. Stain cells in 1 ml of anti-mouse CD45RB FITC, CD25 PE and CD4 APC-Cy7. Mix the cells carefully by pipetting. Leave the tube containing the cell suspension on ice for 20 min without further mixing/inverting/shaking.
16. Wash cells (enriched for CD4⁺ T cells) with PBS/2% HS prior to cell sorting for CD4⁺CD45RB^{hi} T cells on a BD FACSARIA III cell sorter.

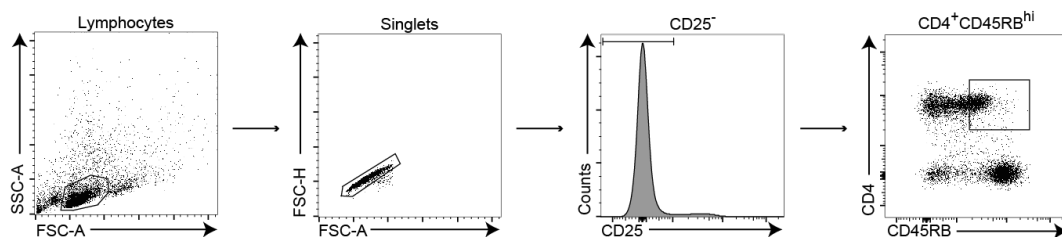


Figure 2. Gating strategy for the selection of naïve CD45RB^{hi} T cells from spleens of donor C57BL/6 congenic mice. Lymphocytes are first selected based on their forward and side scatter properties, followed by the exclusion of doublet cells. Regulatory and activated T cells are excluded by selecting CD25 negative lymphocytes from the singlets gate and finally, naïve CD4⁺ T cells are selected based on their high expression of CD4 and CD45RB.

17. Collect sorted cells in 1 ml of HS (100% HS).
18. Wash sorted CD4⁺CD45RB^{hi} T cells in PBS for 5 min at 370 x g and resuspend in PBS at a concentration of 1 x 10⁶ cells/ml.
Note: Keep the cells on ice at all times. If sorted naïve CD4⁺ T cells cannot be injected right away, keep the cells in HS on ice. Only wash and resuspend the cells with PBS prior to injection.
19. To induce colitis, inject C57BL/6 Rag2^{-/-} mice with 2 x 10⁵ CD4⁺CD45RB^{hi} T cells in a volume of 200 µl intraperitoneally (i.p).
20. Once the animals are colitic (see Procedure B), inject the animals three times with 250-500 µg of anti-CD4 depleting antibody with an interval of 72 h between each injection to induce remission.

Note: Remission induction was initially performed using 500 µg of αCD4 depleting antibody, as described by Brasseit et al., 2016. In our experiments, we can still achieve the same level of CD4⁺ T cell depletion and similar kinetics of remission induction using 3 treatments of 250 µg αCD4 depleting antibody.

21. Monitor the animals daily for weight change and normal activity, with relapse of colonic inflammation expected around 22-25 days (in *Helicobacter* positive recipients) after the first treatment with anti-CD4 depleting antibody.

*Note: To assess the development of colitis, monitor the mice daily since the kinetics of colitis induction is strongly dependent on the composition of the microbiota implying that disease progression may vary from animals of one SPF mouse house facility to the other. In our experience, clinical and histopathological signs of colitis are seen around day 9-10 post T cell transfer in *Helicobacter* positive mice and around day 30 post T cell transfer in *Helicobacter* negative mice. In *Helicobacter* positive recipient mice, clear weight loss (approximately 10%) and appearance of colitogenic CD4⁺ T cells in the blood occurs very rapidly from one day to the other. Moreover, we generally observe that the weight loss correlates strongly with the extent of intestinal inflammation in *Helicobacter* positive but not in *Helicobacter* negative recipients.*

B. Clinical and histopathological scoring

1. Mice with active intestinal inflammation (clinical score ≥ 8) and with detectable CD4⁺ T cells in the blood are considered colitic. For the measurement of CD4⁺ T cells in the blood, collect 2-3 drops of blood by the tail vein in 1 ml of PBS/2% HS/5 mM EDTA. Spin down the cells by centrifugation at 370 x g for 5 min. Resuspend the cells in 100 µl of PBS/2% HS containing anti-mouse CD4 antibody (Refer to Materials and Reagents section for antibody dilution) by pipetting. Following incubation on ice for 15 min, wash the cells in PBS/2% HS and analyse on a flow cytometer.
2. For clinical disease scoring, monitor the following parameters daily:
 - a. Weight loss
 - b. Stool consistency
 - c. Appearance of blood in the stool
 - d. Behavior

Clinical scores	Observations
Weight loss	
0	0%
1	≤5%
2	≤10%
3	≤15%
4	≤20%
Stool consistency	
0	Normal stool consistency
1	Loose stool
2	Diarrhea for 24h
3	Persistent diarrhea
Blood in the stool	
0	No blood
1	Mild signs of blood in feces
3	Readily visible signs of blood in feces
Behavior	
0	Normal
2	Reduced activity
4	Signs of serious pain, reduced activity, hunched appearance and labored breathing

Figure 3. Scoring system used to assess colitic animals following transfer of CD4⁺ CD45RB^{hi} T cells in lymphopenic recipients. Mice with a clinical score of ≥ 8 were considered colitic (multiple combinations leading to a clinical score of 8 are possible).

- For histological disease scoring, collect colon (middle) sections for hematoxylin and eosin (H&E) staining.

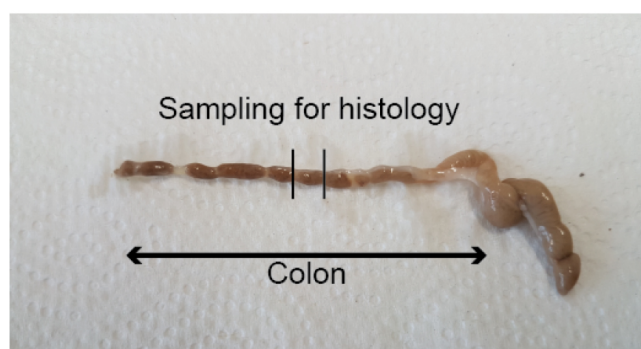


Figure 4. Collection of colon tissue section for histology

- In a blinded manner, measure the following parameters (this should be done by an experienced pathologist). Refer to the publication by Brasseit *et al.*, 2016 in *Mucosal Immunology* for more details:
 - Cellular infiltrates in the lamina propria (Score from 0 to 3)
 - Loss of goblet cells (Score from 0 to 3)

- c. Presence of crypt abscess (Score from 0 to 3)
- d. Epithelial erosion (Score from 0 to 1)
- e. Hyperemia (Score from 0 to 2)
- f. Thickness of the colonic mucosa (Score from 0 to 3)

C. Paraffin embedding and H&E staining

Note: The paraffin embedding and H&E staining are routinely carried out in our histology core lab facility using a fully automated procedure on a Tissue-Tek VIP 6 but the basic steps are described below.

1. Collect 1 cm colonic tissue (middle section) in a uni-cassette® and fix in 4% buffered formalin overnight at room temperature.
2. Process the tissue as follows (using an automated tissue processor):
 - a. 4% buffered formalin for 1.25 h at 35 °C.
 - b. Distilled water for 15 min at room temperature.
 - c. 70% ethanol for 1.5 h at 35 °C.
 - d. 80% ethanol for 1 h at 35 °C.
 - e. 95% ethanol for 1.5 h at 35 °C.
 - f. 100% ethanol for 1 h at 35 °C (three changes of 1 h each).
 - g. Xylene for 1 h at 35 °C (two changes of 1 h each).
 - h. Paraffin for 45 min at 60 °C (four changes of 45 min each).

Note: Changes of ethanol, xylene and paraffin are required to reduce carryover of reagents from previous steps.

3. To embed in a paraffin block. Choose an appropriate mold that corresponds to the size of the cassette.
4. Open the cassette and discard the lid.
5. Add some liquid paraffin into the mold, which is placed on a heating block at 60 °C to prevent the paraffin from solidifying.
6. Transfer the tissue into the mold using warm forceps such that the forceps can be used to transfer the tissue from the cassette to the mold without solidifying the paraffin.
7. Place the mold on a cold plate (-10 °C) to start solidifying the paraffin and immediately press the tissue down with forceps.
8. Place the tissue cassette on top of the mold and press firmly. Ensure that there is sufficient paraffin to cover the plastic cassette.
9. Wait for the paraffin to solidify such that the paraffin block can easily come out of the mold.
10. Cut 5 µm sections using a microtome. Briefly, insert the blade in the holder and adjust the angle of the blade to 5°. Place the paraffin block in the object clamp and adjust to the desired orientation. Cut a few sections (10 µm) to expose the tissue surface. Once the tissue is exposed and the tissue cuts are smooth, adjust the section thickness to 5 µm. Carefully pick up the 5 µm

tissue sections with forceps and float them in a warm water bath at 37 °C. Collect the sections on a clean glass slide and dry on a heating plate at 40 °C for 20 min.

11. To stain with hematoxylin and eosin, first deparaffinize the slides with xylene for 5 min.

12. Rinse twice with xylene and rehydrate the slides by rinsing sequentially in:

- a. Absolute ethanol (two changes)
- b. 95% ethanol
- c. 70% ethanol
- d. Distilled water

Note: The exact volumes are not specified here since this will depend on the size of the glass-staining jar. We recommended that the slides have to be totally submerged in the specified reagent.

13. Stain with Mayer's hemalum solution for 7 min and rinse with tap water.

14. Bleach with 0.5% HCl-ethanol for a few seconds (depending on the desired staining depth of the nucleus) and wash slide immediately with tap water followed by a bluing step in tap water for 10 min.

15. Rinse with 70% ethanol.

16. Counter stain using Eosin-Phloxine solution for 2-3 min. Rinse twice with 70% ethanol.

17. Dehydrate the slides by rinsing sequentially in:

- a. 70% ethanol
- b. 95% ethanol
- c. Absolute ethanol (two changes in total)
- d. Xylene (two changes in total)

Note: The exact volumes are not specified here since this will depend on the size of the glass-staining jar. We recommended that the slides have to be totally submerged in the specified reagent.

18. Mount with Eukitt®

Data analysis

In the design of the experimental set-up, it is recommended to include 4-5 mice/group and perform 2-3 independent experiments. For data analysis, compare recipient mice that received naïve CD45RB^{hi} T cells (experimental group) to those that received CD45RB^{lo} T cells (control group, no colitis). To assess the statistical significance, use an unpaired *t*-test on GraphPad Prism 6 (La Jolla, CA).

Notes

1. To ensure the best cell yield and viability, always work on ice unless indicated otherwise.

2. To get rid of the supernatant following centrifugation, aspirate the supernatant and discard rather than pouring it off since cells may not adhere strongly to the bottom of the 5 ml PS tube.
3. For cell resuspension, this should be performed gently using a pipette and not by vortexing.
4. Prior to cell injection, mix the cells by inverting the tube up and down to ensure that all mice are injected with the same number of cells.
5. For intraperitoneal injection, ensure that the needle is inserted properly in the peritoneal cavity prior to injecting the cells.
6. If *Rag*^{-/-} recipients are imported from another mouse house facility, it is highly advisable to house these mice for at least two weeks prior to naïve CD4⁺ T cell transfer to allow stabilization of the microbiota.

Recipes

1. Phosphate-buffered saline (PBS)
137 mM NaCl
1.69 mM Na₂HPO₄·2H₂O
8.63 mM K₂HPO₄·3H₂O
2. ACK red blood lysis buffer
150 mM NH₄Cl
10 mM KHCO₃
0.1 mM EDTA
3. Eosin-Phloxine solution
100 ml of 1% eosin stock solution
10 ml of 1% phloxine stock solution
780 ml of 95% ethanol
4 ml glacial acetic acid
4. 0.5% HCl-ethanol
20 ml of 25% hydrochloric acid
980 ml of 70% ethanol

Acknowledgments

This work was supported by the Swiss National Science Foundation Sinergia grant to Andrew Macpherson, Christoph Mueller, Wolf-Dieter Hardt and Daniela Finke as well as by the SNSF grant No. 31-138392 to C.M. The described protocol was first outlined in Brasseit *et al.*, 2016.

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