

Analysis of FoxP3⁺ regulatory T cells from murine colons

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Background

The forkhead box transcription factor 3 (FoxP3) has an important function in the development of immunosuppressive activities of regulatory (Treg) cells^{1,2,3} and represents a specific marker for these cells. FoxP3⁺ Treg cells play a vital role in maintaining the immune homeostasis, especially in the largest immune organ, the intestine, where a very high concentration of particular antigens from commensals and ingested food is present⁴.

Many different methods are available for dissociating cells from intestines with home-brew enzyme mixtures. However, those protocols are labor-intensive and the results can vary between operators. For that reason, the validity of analysis of cell populations from intestine largely depends on the preparation methods used and the operator bias introduced. The gentleMACS™ Dissociator and the Lamina Propria Dissociation Kit, mouse provide the opportunity to standardize the procedure and generate reproducible results. The gentleMACS Protocol combines automated mechanical tissue dissociation on the gentleMACS Dissociator with enzymatic dissociation based on a pre-defined enzyme mix. In this study, we compared the cell yield obtained with a manual protocol and the standard gentleMACS Protocol to identify FoxP3⁺ Treg cells in murine intestines.

Material and methods

Material

- Wild-type BALB/c mice, 8 weeks old
- gentleMACS Dissociator
- gentleMACS C Tubes
- Lamina Propria Dissociation Kit, mouse
- MACSmix™ Tube Rotator in combination with an incubator at 37 °C.
- Cell filters (100 µm)
- HBSS (w): Hank's balanced salt solution (HBSS) with Ca²⁺ and Mg²⁺ containing 10 mM HEPES
- HBSS (w/o): HBSS without Ca²⁺ and Mg²⁺ containing 10 mM HEPES
- EDTA
- EasyColl™
- Collagenase D
- DNase I
- Dispase
- Fetal bovine serum (FBS)
- Dithiothreitol (DTT)
- PB buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, with 0.5% bovine serum albumin (BSA)

Methods

1. Dissect the mice and prepare mouse colons.
2. Remove feces from colons by forceps and flushing with HBSS (w/o).
3. Open the colons longitudinally and cut them into pieces of approximately 0.5 cm in length.
4. Transfer the colon pieces into a 50 mL tube.

5. Add pre-digestion solutions (20 mL 1× HBSS (w/o) containing 5 mM EDTA, 5% FBS, 1 mM DTT) and incubate for 20 minutes at 37 °C under continuous rotation using the MACSmix™ Tube Rotator.
6. Vortex for 10 seconds and remove the supernatant.
7. Repeat steps 5 and 6 twice and discard the supernatant containing intraepithelial cells.
8. Transfer the tissues to a new 50 mL tube and wash the tissues with 10 mL HBSS (w), 10% FBS to remove EDTA.
9. Prepare tissues for lamina propria dissociation as follows:
 - gentleMACS Protocol: transfer tissues into gentleMACS C Tubes and add enzyme mix by following the data sheet of the Lamina Propria Dissociation Kit, mouse.
 - Manual method: cut the tissues into smaller pieces and incubate with 5 mL HBSS (w), 10% FBS, Collagenase D (1 mg/mL), DNase I (0.1 mg/mL), and Dispase (0.1 U/mL) per intestine at 37 °C for 45 minutes.
10. Apply digested tissue onto a 100 µm strainer placed on a 50 mL tube and wash with 10 mL of PB buffer.
11. Centrifuge at 300×g for 10 minutes.
12. Resuspend cell pellets in PB buffer.
13. Cells are further separated using a 40–70% Easycoll gradient at 780×g without brake at room temperature for 20 minutes. After centrifugation, lymphocytes are isolated from the interphase.
14. Spin down and wash the cells with PB buffer.
15. Stain isolated cells with anti-CD3, anti-CD4, anti-FoxP3, and anti-Neuropilin-1 (Nrp1) antibodies and analyze with a flow cytometer.

Results

Comparison of Treg cell numbers obtained with the gentleMACS™ Protocol or the manual method

Lamina propria cells from mouse colons were prepared using the gentleMACS™ Dissociator in combination with the Lamina Propria Dissociation Kit, mouse or a manual method based on a mix of collagenase and dispase. The numbers of viable CD3⁺CD4⁺FoxP3⁺ Treg cells were determined by flow cytometry (fig. 1A). Compared to the manual method, the gentleMACS Protocol resulted in a 3-fold higher number of FoxP3⁺ Treg cells from colons (fig. 1B).

Flow cytometry analysis of Treg cells obtained with the two methods

We compared the levels of Nrp1^{5,6} expression among the Treg cells, as Nrp1 on the cell surface can be sensitive to proteolysis caused by crude enzyme components (data not shown). The expression levels of Nrp1 in colonic Treg cells were comparable between the two methods (fig. 2). This suggests that the Nrp1 epitope was preserved during the enzymatic tissue dissociation process.

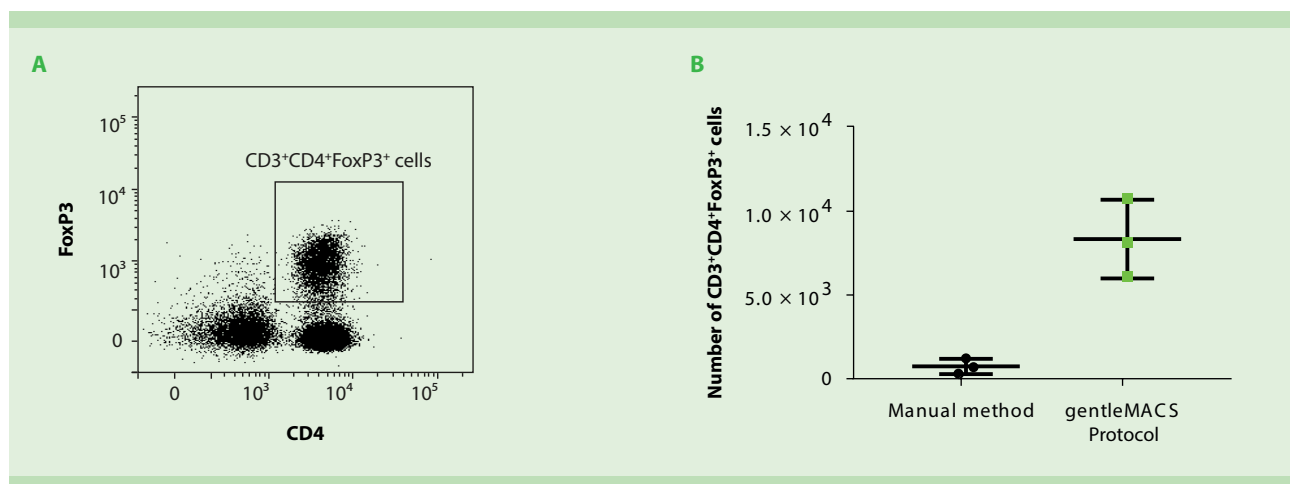


Figure 1: Analysis of CD4⁺FoxP3⁺ Treg cells in cell suspensions of lamina propria from mouse colons by flow cytometry using Flowlogic™ software. (A) Representative flow cytometry data showing FoxP3 versus CD4 expression, gated on CD3⁺ lymphocytes. Dead cells were excluded from the analysis. (B) Comparison of numbers of viable CD3⁺CD4⁺FoxP3⁺ Treg cells in colonic lamina propria, obtained with the gentleMACS Protocol or the manual method. (n = 3 per group)

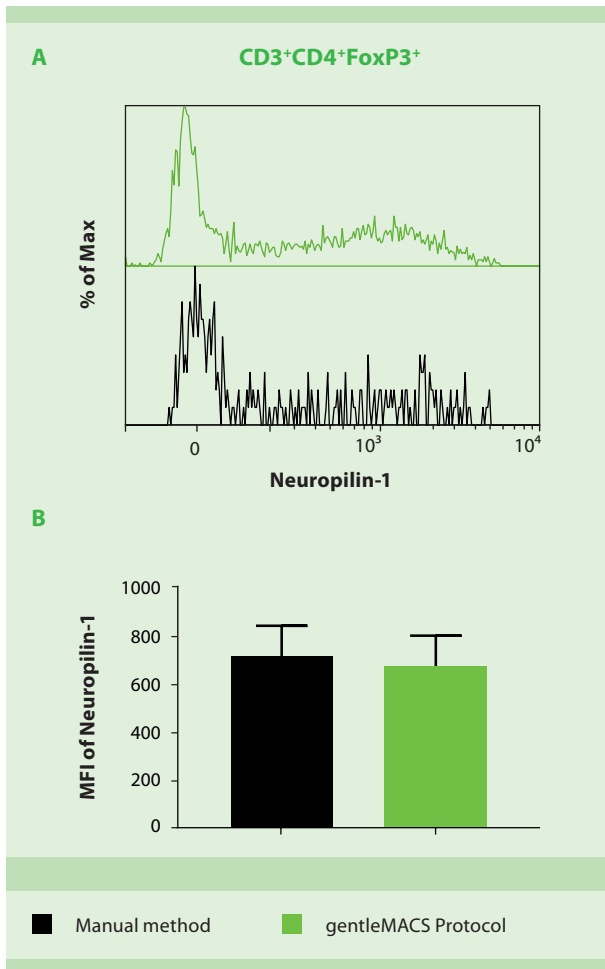


Figure 2: Comparison of Neuropilin-1 expression on colonic CD4⁺FoxP3⁺ Treg cells, obtained with the gentleMACS Protocol (green) or the manual method (black). (A) Representative histograms showing expression levels of Neuropilin-1 on colonic CD4⁺FoxP3⁺ Treg cells. (B) Mean fluorescence intensity (MFI) of Nrp1 on colonic CD4⁺FoxP3⁺ Treg cells. Data were gated on CD3⁺CD4⁺FoxP3⁺ cells. (n = 2, per group)

Conclusion

Preparing cells from murine colons has been challenging as the commonly used protocol is labor intensive and results in low cell yield. Using the gentleMACS™ Dissociator in combination with the Lamina Propria Dissociation Kit, mouse enabled us to standardize the workflow and obtain consistently higher number of CD4⁺FoxP3⁺ Treg cells. This allows the generation of a sufficient number of cells from murine colons for the analysis of Treg cell subpopulations.

References

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