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Detection of iNOS/TNF in cells of the small intestinal lamina propria by FACS

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Abstract

The largest mucosal surface in the body is in the gastrointestinal (GI) tract, a location that is heavily colonized by normally harmless microbes. A key mechanism required for maintaining a homeostatic balance between this microbial burden and the lymphocytes that densely populate the GI tract is the production and trans-epithelial transport of poly-reactive IgA1. Within the mucosal tissues, B cells respond to cytokines, sometimes in the absence of T cell help, undergo class switch recombination (CSR) of their Immunoglobulin (Ig) receptor to IgA, and differentiate to become plasma cells (PC)2. However, IgA-secreting PC likely have additional attributes that are needed for coping with the tremendous bacterial load in the GI tract. Here we describe a detailed method to characterize IgA+B220lowCD11clowiNOS+TNFα+ cells that we named TNFα-iNOS-producing (TiP)-PC in the lamina propria of mice by FACS.

Introduction

The largest mucosal surface in the body is in the gastrointestinal (GI) tract, a location that is heavily colonized by normally harmless microbes. A key mechanism required for maintaining a homeostatic balance between this microbial burden and the lymphocytes that densely populate the GI tract is the production and trans-epithelial transport of poly-reactive IgA1. Within the mucosal tissues, B cells respond to cytokines, sometimes in the absence of T cell help, undergo class switch recombination (CSR) of their Immunoglobulin (Ig) receptor to IgA, and differentiate to become plasma cells (PC)2. However, IgA-secreting PC likely have additional attributes that are needed for coping with the tremendous bacterial load in the GI tract. Here we describe a detailed method to characterize IgA+B220lowCD11clowiNOS+TNFα+ cells that we named TNFα-iNOS-producing (TiP)-PC in the lamina propria of mice by FACS.

Reagents

Wash intestine buffer (WIB): HBSS (Gibco), 2%FBS (heat inactivated), 15mM hepes (Gibco), keep cold on ice

Intestinal EDTA Buffer: HBSS, 10%FBS, 15mM hepes and 5mM EDTA, keep at room temp.

Digestion buffer: RPMI (Gibco), 10%FBS, 15mM hepes pre warm (37oC)

Petri Dishes

50 ml falcon tubes

15ml falcon tubes

Kit for dissection

5ml syringes

Oral gavage needle

Gauze

Cell Strainer 70µm (BD)

Microtest Plates 96 well V-Bottom (Sarstedt)

5ml polystyrene round bottom tubes (BD)

Sterile PBS 1x (Gibco)

Fetal Bovine Serum (FBS) (Gibco)

FACS Buffer: PBS/2% FBS

Fixation Permeabilization Kit (BD Biosciences)

LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen)

BD Compensation beads (Anti Rat and Anti Hamster Ig)

Monoclonal antibodies:

Antibody	Company	Clone
Hamster Anti mouse CD11c PECy7	eBioscience	N418
Rat Anti mouse B220 eF450	eBioscience	RA3-6B2
Rat anti mouse IgA FITC	Southern Biotech	11-44-2
Anti mouse monoclonal NOS2 AF647	Santa Cruz Biotecnology	C-11
Rat anti mouse TNFa PE	eBioscience	MP6-XT22
Mouse IgG1 Isotype AF647	Santa Cruz Biotecnology	
Rat IgG1 Isotype PE	eBioscience	

Equipment

13 -colour BD LSR II flow cytometer

FlowJo Software 8.8

Allegra 6R Centrifuge (Beckman Coulter)

Vortex

Shaking plataform

Procedure

Isolation of lamina propria \(LP) cells from small intestine:

1. Remove small intestine between stomach and cecum. Keep the intestine in a 50ml tube with wash intestine buffer \(WIB) on ice, try to remove gentlyadditional tissue or fat.

2. Flush gut with 10ml cold WIB using gavage needle.

3. In petri dish with WIB remove remaining mesenteric tissue and fat with forceps, using light from a Rx projector.

4. Locate remaining Peyer’s patches \(PPs). Excise by pinching PPs with forceps and cutting as close as possible with scissors.

5. Use flat part of scissors to gently expel remaining mucus and feces.

6. In new petri dish containing WIB open intestine along the length with scissors.

7. Cut gut into small pieces \(5mm) in to a 50ml conical tube with 20ml of cold WIB.

8. Invert several times, vortexing and pour off supernantant. Refill tube and repeat 2 more times.

9. Transfer gut pieces to 50ml falcon tubes containing 20ml pre warm \(37^{O}C) intestinal EDTA buffer.

10. Vortex and Incubate with shaking platform for 20 minutes at 37^{O}C

11. Vortex tube on high for 15 seconds.

12. Allow pieces to settle. Discard supernatant in waste \(this supernantant contains intraepithelial lymphocytes).

13. Add 20ml of intestinal EDTA buffer and repeat steps 10-12 at least twice until clear.

14. Put pieces of tissue in a gauze and wash pieces with WIB twice. EDTA will inhibit collagenase so you must be sure to remove remaining EDTA beforeproceeding.

15. Transfer pieces to 50ml falcon tubes containing 20ml digestion buffer and add 5mg of collagenase 4 \(Sigma) and 0.5mg of DNaseI \(Roche). CAUTION:Concentration of collagenase 4 can be variable even between lots. A titration should be done to determine the optimal concentration to use withoutimpacting cell viability.

16. Incubate in water bath 1 hr at 37^{O}C. Vortex tube every 15 minutes \(until the tissue disappear).

17. Pour supernatant through 70um nylon filter in to 50ml tube.

Once LP cells are isolated, we proceed to do staining in four main steps: 1. viability staining, 2.

surface marker staining, 3.fixation/permeabilization and 4. intracellular staining, as follows:

CAUTION: You should use PBS only \(without proteins) as a buffer during viability staining.

1. Wash the cells with 5 ml of PBS

2. Spin at 1200 rpm for 10 min at 4 °C

3. Repeat steps 1 and 2

4. Resuspend cells \(1-10 million) in to 1ml of PBS

5. Add 1ml of Aqua and mix well

6. Incubate at 4 °C for 30 min, protected from the light

7. Add 1 ml of PBS to wash the cells

8. Spin at 1200 rpm for 10 min at 4 °C

9. Repeat steps 7 and 8 once

- Surface staining

10. Transfer cells into a 96 V well plate. Ensure that you divide cell suspensions into two wells \(specific and isotype control staining) per sample.Also, you will need some wells for unstained control, Aqua staining only and fluorescence minus one \(FMO) controls for IgA, B220 as well as CD11cstains. For some intra-cellular stains, isotype controls are “stricter” than FMO. These were used to gauge the level of TNF/iNOS staining.

11. Add 50ml/well of the following surface antibody cocktail \(prepare the cocktail in FACS Buffer):

Antibody	Dilution
rat anti-mouse CD16/CD32 \(Fc Block Clone:2.4G2) Hybridoma supernatant	1/10
CD11c PECy7	1/100
B220 EF450	1/300

12. Incubate at 4 °C for 30 min, protected from the light

13. Wash 2x in FACS Buffer, spin at 1200 rpm for 5 min at 4 °C

-Fixation/permeabilization

14. Resuspend cells in 100 ml/well Cytofix/Cytoperm

15. Incubate for 20 min at 4 °C

15. Wash 2x in 1x Perm/wash \(diluted in water)

-Intracellular staining

16. Resuspend in 50 ml of intracellular staining cocktail \(prepare the cocktail in Perm/wash buffer):

CAUTION: Spin the cocktail 5000 rpm for 10 min before you use it.

Specific Staining	Dilution	Isotype Staining	Dilution
IgA FITC	1/100	N/A \(FMO used)	1/100
NOS2 AF647	1/25	AF647 Mouse IgG1 Isotype	1/25
TNF PE	1/400	PE Rat IgG1 Isotype	1/400

17. Incubate for 20 min at 4 °C

18. Wash 2x in 1x Perm/wash \(diluted in water)

19. Re-suspend cells in 200 ml of FACS buffer.

20. Store at 4˚C until ready to run FACS.

21. Acquire samples in a LSRII machine. We use BD Compensation beads for compensation as well as application settings to enhance reproducibilitybetween experiments.

Timing

Once isolated, the staining procedure of lamina propria cells takes approximately three hours.

Troubleshooting

1. There are several antibodies available to identify iNOS by FACS. As some of the polyclonal antibodies gave high background staining we recommend applying monoclonal antibodies. We have tested several of them, observing the cleanest signal with AF647 NOS2 (C-11), which is a monoclonal antibody. Although, the background with this antibody is lower, the background can be variable so it is important to include the isotype staining at the same time for each sample and subtract the background from the specific staining for analysis.
2. It is really important to titrate the iNOS antibodies used carefully even between lots as we have experienced differences. The shelf-life for the Ab is approximately 3 months.
3. Spinning the intracellular cocktail before you use it helps to minimize the background.

Anticipated Results

The viability should be over 60%, otherwise the frequency of IgA+iNOS+ may be dramatically

decreased since IgA+ PC appear to be very susceptible to processing-related death.

The mean frequency of IgA+B220low cells is around 14%. From this IgA+B220low cells about 3-5% are iNOS+ and 1% are iNOS+TNF+ double-positive, so you need to acquire at least 2 million of total lymphocytes in order to have enough cells to analyze.

References

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Figures

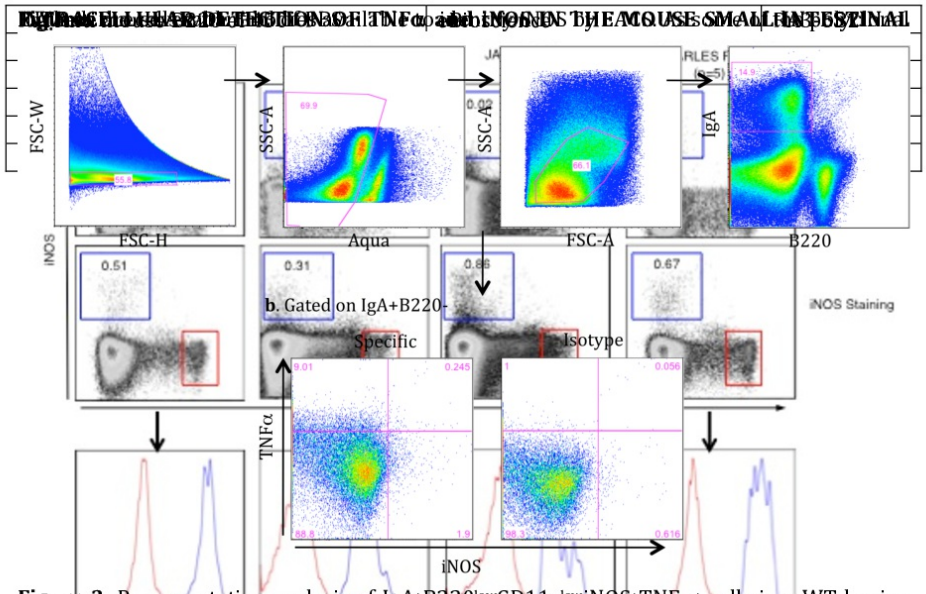

Figure 2. Representative analysis of IgA⁺B220^{low}CD11c^{low}iNOS⁺TNFα⁺ cells in a WT lamina propria by FACS. A. Systematic analysis on singlet, viable lymphocytes that are IgA⁺B220^{low} B. Gating on IgA⁺B220^{low} cells, we analyzed expression of iNOS and TNFα .

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Conflict of interest

The authors declare no competing financial interest.

Abstract

Isolation of lymphocytes from the small intestinal lamina propria (LP) is a prerequisite for many types of mucosal immunological analysis. Here we describe a method for the isolation of LP cells from murine small intestine that yields a high number of viable cells suitable for flow cytometric analysis. Briefly, intestinal epithelial cells and intraepithelial lymphocytes are removed by incubation in EDTA and DTT containing buffer. The remaining tissue is then digested with collagenase and DNase I to release LP cells. Once isolated, the staining procedure of lamina propria cells takes approximately three hours. We proceed with staining in four main steps: 1. viability staining, 2. surface marker staining, 3. fixation/permeabilization and 4. intracellular staining, as described below. Using this protocol, we routinely obtain 1-4 x 10^6 LP cells from a single small intestine. Importantly, this protocol can be used to isolate LP cells from mice of different ages without impacting cell viability.

Introduction

The small intestinal lamina propria (LP) is a mucosal tissue that is rich in immune cells. Many of these cells, including IgA plasma cells, dendritic cells, macrophages, innate lymphoid cells and T cells, play important roles in maintaining intestinal homeostasis and in protecting the host from pathogens (Brandtzaeg, 2011; Cerutti and Rescigno, 2008; Macpherson et al., 2008; Mowat, 2003; Sansonetti, 2011; Vallance and Finlay, 2000). Thus, the ability to isolate and characterize LP cells is important for understanding the mechanisms that regulate intestinal immunity and inflammation. Here we describe a protocol for the isolation of LP cells from murine small intestine that yields a high number of viable cells suitable for flow cytometric analysis. Briefly, intestinal epithelial cells and intraepithelial lymphocytes are removed by incubation in EDTA and DTT containing buffer. The remaining tissue is then digested with collagenase and DNase I to release LP cells. Once isolated, the staining procedure of lamina propria cells takes approximately three hours. We proceed with staining in four main steps: 1. viability staining, 2. surface marker staining, 3. fixation/permeabilization and 4. intracellular staining, as described below. Using this protocol, we routinely obtain 1-4 x 10^6 LP cells from a single small intestine. Importantly, this protocol can be used to isolate LP cells from mice of different ages without impacting cell viability.

Materials and Reagents

1. Fetal bovine serum (FBS) (Hyclone, Cat# SH30071.03)
2. Dithiothreitol (DTT) (Sigma, Cat# D9779)
3. Ethylenediaminetetraacetic acid (EDTA) (Sigma, Cat# E7889)
4. Collagenase D (Roche, Cat# 11088866001)
5. DNase I (Roche, Cat# 10104159001)
6. Hanks' Balanced Salt Solution (HBSS) (Invitrogen, Cat# 14175-095)
7. RPMI 1640 (Invitrogen, Cat# 11875-093)
8. Phosphate buffered saline (PBS) (Invitrogen, Cat# 14190-144)
9. Penicillin-Streptomycin (Pen-Strep) (Invitrogen, Cat# 15140-122)
10. Sodium Bicarbonate (Sigma, Cat# S5761)
11. HEPES (Sigma, Cat# H3375)
12. Sodium Hydroxide (NaOH) (Sigma, Cat# S5881)
13. Hydrochloric acid (HCl) (Sigma, Cat# H1758)
14. Sodium Azide (Sigma, Cat# S2002)
15. LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen)

Monoclonal antibodies:

Antibody	Company	Clone
CD45	eBioscience	30-F11
CD3	eBioscience	145-2C11
CD4	eBioscience	RM4-5
CD8	eBioscience	53-6.7
CD19	eBioscience	1D3
B220	eBioscience	RA3-6B2
CD11c	eBioscience	N418

Any monoclonal antibodies specific for murine cell surface markers can be used in this protocol. Antibodies should be titrated for optimal performance prior to use in this protocol. Titration should be done to determine the optimal concentration of antibody to use that will yield the highest signal to noise ratio. Titration should be done using cells and staining buffer that will be used in the actual experiment. Compensation beads for compensation of multicolor FACS.

Equipment

1. Tissue culture incubator (37°C, 5% CO$_2$)
2. Refrigerated benchtop centrifuge
3. Shaking incubator (37°C, Vortex-Brine Model I2500)
4. Vortex
5. Timer
6. Scissors
7. Forceps
8. Cell strainer (70 µm nylon mesh, BD Falcon, Cat# 352350)
9. 6 well plate
10. 50ml tube
11. Gauze
12. Flow cytometer (LSR II, BD Biosciences)

Procedure

1. Remove small intestine from mouse and place in 50ml tube.
2. Remove Peyer's patches from small intestine.
3. Open intestine longitudinally and cut into 1cm pieces.
4. Wash tissue 1 time with 30ml PBS/2% FBS.
5. Incubate tissue in 30ml PBS/2% FBS, 5mM EDTA, 1mM DTT for 20 minutes at 37°C, 250 rpm in shaking incubator.
6. Pour tissue and buffer through sieve and discard flow through.
7. Repeat step 5.
8. Pour tissue and buffer through sieve and discard flow through.
9. Wash tissue 1 time with 30ml PBS/2% FBS.
10. Transfer tissue to 50ml falcon tubes containing 20ml digestion buffer and add 5mg of collagenase and 1mg of DNase I (Roche). CAUTION: Concentration of collagenase and DNase I may vary between lots. Titration should be done to determine the optimal concentration of collagenase and DNase I for each lot.
11. Incubate tissue in shaking incubator (37°C, 250 rpm) for 15 minutes.
12. Vortex tube for 15 seconds.
13. Repeat steps 11 and 12 two more times.
14. Pour tissue and digestion buffer through sieve and discard flow through.
15. Wash tissue 1 time with 30ml PBS/2% FBS.
16. Pour tissue and buffer through sieve and discard flow through.
17. Wash tissue 1 time with 30ml PBS/2% FBS.
18. Pour tissue and buffer through sieve and discard flow through.
19. Wash tissue 1 time with 30ml PBS/2% FBS.
20. Pour tissue and buffer through sieve and discard flow through.
21. Wash tissue 1 time with 30ml PBS/2% FBS.
22. Pour tissue and buffer through sieve and discard flow through.
23. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
24. Mince tissue with scissors.
25. Transfer tissue and buffer through sieve and discard flow through.
26. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
27. Shake tube by hand for 10 seconds.
28. Pour tissue and buffer through sieve and discard flow through.
29. Repeat steps 26 to 28 at least twice until the buffer is clear of mucus.
30. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
31. Mince tissue with scissors.
32. Transfer tissue and buffer through sieve and discard flow through.
33. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
34. Shake tube by hand for 10 seconds.
35. Pour tissue and buffer through sieve and discard flow through.
36. Repeat steps 33 to 35 at least twice until the buffer is clear of mucus.
37. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
38. Mince tissue with scissors.
39. Transfer tissue and buffer through sieve and discard flow through.
40. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
41. Shake tube by hand for 10 seconds.
42. Pour tissue and buffer through sieve and discard flow through.
43. Repeat steps 40 to 42 at least twice until the buffer is clear of mucus.
44. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
45. Mince tissue with scissors.
46. Transfer tissue and buffer through sieve and discard flow through.
47. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
48. Shake tube by hand for 10 seconds.
49. Pour tissue and buffer through sieve and discard flow through.
50. Repeat steps 47 to 49 at least twice until the buffer is clear of mucus.
51. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
52. Mince tissue with scissors.
53. Transfer tissue and buffer through sieve and discard flow through.
54. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
55. Shake tube by hand for 10 seconds.
56. Pour tissue and buffer through sieve and discard flow through.
57. Repeat steps 54 to 56 at least twice until the buffer is clear of mucus.
58. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
59. Mince tissue with scissors.
60. Transfer tissue and buffer through sieve and discard flow through.
61. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
62. Shake tube by hand for 10 seconds.
63. Pour tissue and buffer through sieve and discard flow through.
64. Repeat steps 61 to 63 at least twice until the buffer is clear of mucus.
65. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
66. Mince tissue with scissors.
67. Transfer tissue and buffer through sieve and discard flow through.
68. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
69. Shake tube by hand for 10 seconds.
70. Pour tissue and buffer through sieve and discard flow through.
71. Repeat steps 68 to 70 at least twice until the buffer is clear of mucus.
72. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
73. Mince tissue with scissors.
74. Transfer tissue and buffer through sieve and discard flow through.
75. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
76. Shake tube by hand for 10 seconds.
77. Pour tissue and buffer through sieve and discard flow through.
78. Repeat steps 75 to 77 at least twice until the buffer is clear of mucus.
79. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
80. Mince tissue with scissors.
81. Transfer tissue and buffer through sieve and discard flow through.
82. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
83. Shake tube by hand for 10 seconds.
84. Pour tissue and buffer through sieve and discard flow through.
85. Repeat steps 82 to 84 at least twice until the buffer is clear of mucus.
86. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
87. Mince tissue with scissors.
88. Transfer tissue and buffer through sieve and discard flow through.
89. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
90. Shake tube by hand for 10 seconds.
91. Pour tissue and buffer through sieve and discard flow through.
92. Repeat steps 89 to 91 at least twice until the buffer is clear of mucus.
93. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
94. Mince tissue with scissors.
95. Transfer tissue and buffer through sieve and discard flow through.
96. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
97. Shake tube by hand for 10 seconds.
98. Pour tissue and buffer through sieve and discard flow through.
99. Repeat steps 96 to 98 at least twice until the buffer is clear of mucus.
100. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
101. Mince tissue with scissors.
102. Transfer tissue and buffer through sieve and discard flow through.
103. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
104. Shake tube by hand for 10 seconds.
105. Pour tissue and buffer through sieve and discard flow through.
106. Repeat steps 103 to 105 at least twice until the buffer is clear of mucus.
107. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
108. Mince tissue with scissors.
109. Transfer tissue and buffer through sieve and discard flow through.
110. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
111. Shake tube by hand for 10 seconds.
112. Pour tissue and buffer through sieve and discard flow through.
113. Repeat steps 110 to 112 at least twice until the buffer is clear of mucus.
114. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
115. Mince tissue with scissors.
116. Transfer tissue and buffer through sieve and discard flow through.
117. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
118. Shake tube by hand for 10 seconds.
119. Pour tissue and buffer through sieve and discard flow through.
120. Repeat steps 117 to 119 at least twice until the buffer is clear of mucus.
121. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
122. Mince tissue with scissors.
123. Transfer tissue and buffer through sieve and discard flow through.
124. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
125. Shake tube by hand for 10 seconds.
126. Pour tissue and buffer through sieve and discard flow through.
127. Repeat steps 124 to 126 at least twice until the buffer is clear of mucus.
128. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
129. Mince tissue with scissors.
130. Transfer tissue and buffer through sieve and discard flow through.
131. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
132. Shake tube by hand for 10 seconds.
133. Pour tissue and buffer through sieve and discard flow through.
134. Repeat steps 131 to 133 at least twice until the buffer is clear of mucus.
135. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
136. Mince tissue with scissors.
137. Transfer tissue and buffer through sieve and discard flow through.
138. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
139. Shake tube by hand for 10 seconds.
140. Pour tissue and buffer through sieve and discard flow through.
141. Repeat steps 138 to 140 at least twice until the buffer is clear of mucus.
142. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
143. Mince tissue with scissors.
144. Transfer tissue and buffer through sieve and discard flow through.
145. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
146. Shake tube by hand for 10 seconds.
147. Pour tissue and buffer through sieve and discard flow through.
148. Repeat steps 145 to 147 at least twice until the buffer is clear of mucus.
149. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
150. Mince tissue with scissors.
151. Transfer tissue and buffer through sieve and discard flow through.
152. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
153. Shake tube by hand for 10 seconds.
154. Pour tissue and buffer through sieve and discard flow through.
155. Repeat steps 152 to 154 at least twice until the buffer is clear of mucus.
156. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
157. Mince tissue with scissors.
158. Transfer tissue and buffer through sieve and discard flow through.
159. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
160. Shake tube by hand for 10 seconds.
161. Pour tissue and buffer through sieve and discard flow through.
162. Repeat steps 159 to 161 at least twice until the buffer is clear of mucus.
163. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
164. Mince tissue with scissors.
165. Transfer tissue and buffer through sieve and discard flow through.
166. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
167. Shake tube by hand for 10 seconds.
168. Pour tissue and buffer through sieve and discard flow through.
169. Repeat steps 166 to 168 at least twice until the buffer is clear of mucus.
170. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
171. Mince tissue with scissors.
172. Transfer tissue and buffer through sieve and discard flow through.
173. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
174. Shake tube by hand for 10 seconds.
175. Pour tissue and buffer through sieve and discard flow through.
176. Repeat steps 173 to 175 at least twice until the buffer is clear of mucus.
177. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
178. Mince tissue with scissors.
179. Transfer tissue and buffer through sieve and discard flow through.
180. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
181. Shake tube by hand for 10 seconds.
182. Pour tissue and buffer through sieve and discard flow through.
183. Repeat steps 180 to 182 at least twice until the buffer is clear of mucus.
184. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
185. Mince tissue with scissors.
186. Transfer tissue and buffer through sieve and discard flow through.
187. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
188. Shake tube by hand for 10 seconds.
189. Pour tissue and buffer through sieve and discard flow through.
190. Repeat steps 187 to 189 at least twice until the buffer is clear of mucus.
191. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
192. Mince tissue with scissors.
193. Transfer tissue and buffer through sieve and discard flow through.
194. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
195. Shake tube by hand for 10 seconds.
196. Pour tissue and buffer through sieve and discard flow through.
197. Repeat steps 194 to 196 at least twice until the buffer is clear of mucus.
198. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
199. Mince tissue with scissors.
200. Transfer tissue and buffer through sieve and discard flow through.
201. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
202. Shake tube by hand for 10 seconds.
203. Pour tissue and buffer through sieve and discard flow through.
204. Repeat steps 201 to 203 at least twice until the buffer is clear of mucus.
205. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
206. Mince tissue with scissors.
207. Transfer tissue and buffer through sieve and discard flow through.
208. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
209. Shake tube by hand for 10 seconds.
210. Pour tissue and buffer through sieve and discard flow through.
211. Repeat steps 208 to 210 at least twice until the buffer is clear of mucus.
212. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
213. Mince tissue with scissors.
214. Transfer tissue and buffer through sieve and discard flow through.
215. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
216. Shake tube by hand for 10 seconds.
217. Pour tissue and buffer through sieve and discard flow through.
218. Repeat steps 215 to 217 at least twice until the buffer is clear of mucus.
219. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
220. Mince tissue with scissors.
221. Transfer tissue and buffer through sieve and discard flow through.
222. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
223. Shake tube by hand for 10 seconds.
224. Pour tissue and buffer through sieve and discard flow through.
225. Repeat steps 222 to 224 at least twice until the buffer is clear of mucus.
226. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
227. Mince tissue with scissors.
228. Transfer tissue and buffer through sieve and discard flow through.
229. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
230. Shake tube by hand for 10 seconds.
231. Pour tissue and buffer through sieve and discard flow through.
232. Repeat steps 229 to 231 at least twice until the buffer is clear of mucus.
233. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
234. Mince tissue with scissors.
235. Transfer tissue and buffer through sieve and discard flow through.
236. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
237. Shake tube by hand for 10 seconds.
238. Pour tissue and buffer through sieve and discard flow through.
239. Repeat steps 236 to 238 at least twice until the buffer is clear of mucus.
240. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
241. Mince tissue with scissors.
242. Transfer tissue and buffer through sieve and discard flow through.
243. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
244. Shake tube by hand for 10 seconds.
245. Pour tissue and buffer through sieve and discard flow through.
246. Repeat steps 243 to 245 at least twice until the buffer is clear of mucus.
247. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
248. Mince tissue with scissors.
249. Transfer tissue and buffer through sieve and discard flow through.
250. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
251. Shake tube by hand for 10 seconds.
252. Pour tissue and buffer through sieve and discard flow through.
253. Repeat steps 250 to 252 at least twice until the buffer is clear of mucus.
254. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
255. Mince tissue with scissors.
256. Transfer tissue and buffer through sieve and discard flow through.
257. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
258. Shake tube by hand for 10 seconds.
259. Pour tissue and buffer through sieve and discard flow through.
260. Repeat steps 257 to 259 at least twice until the buffer is clear of mucus.
261. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
262. Mince tissue with scissors.
263. Transfer tissue and buffer through sieve and discard flow through.
264. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
265. Shake tube by hand for 10 seconds.
266. Pour tissue and buffer through sieve and discard flow through.
267. Repeat steps 264 to 266 at least twice until the buffer is clear of mucus.
268. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
269. Mince tissue with scissors.
270. Transfer tissue and buffer through sieve and discard flow through.
271. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
272. Shake tube by hand for 10 seconds.
273. Pour tissue and buffer through sieve and discard flow through.
274. Repeat steps 271 to 273 at least twice until the buffer is clear of mucus.
275. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
276. Mince tissue with scissors.
277. Transfer tissue and buffer through sieve and discard flow through.
278. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
279. Shake tube by hand for 10 seconds.
280. Pour tissue and buffer through sieve and discard flow through.
281. Repeat steps 278 to 280 at least twice until the buffer is clear of mucus.
282. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
283. Mince tissue with scissors.
284. Transfer tissue and buffer through sieve and discard flow through.
285. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
286. Shake tube by hand for 10 seconds.
287. Pour tissue and buffer through sieve and discard flow through.
288. Repeat steps 285 to 287 at least twice until the buffer is clear of mucus.
289. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
290. Mince tissue with scissors.
291. Transfer tissue and buffer through sieve and discard flow through.
292. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
293. Shake tube by hand for 10 seconds.
294. Pour tissue and buffer through sieve and discard flow through.
295. Repeat steps 292 to 294 at least twice until the buffer is clear of mucus.
296. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
297. Mince tissue with scissors.
298. Transfer tissue and buffer through sieve and discard flow through.
299. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
300. Shake tube by hand for 10 seconds.
301. Pour tissue and buffer through sieve and discard flow through.
302. Repeat steps 299 to 301 at least twice until the buffer is clear of mucus.
303. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
304. Mince tissue with scissors.
305. Transfer tissue and buffer through sieve and discard flow through.
306. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
307. Shake tube by hand for 10 seconds.
308. Pour tissue and buffer through sieve and discard flow through.
309. Repeat steps 306 to 308 at least twice until the buffer is clear of mucus.
310. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
311. Mince tissue with scissors.
312. Transfer tissue and buffer through sieve and discard flow through.
313. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
314. Shake tube by hand for 10 seconds.
315. Pour tissue and buffer through sieve and discard flow through.
316. Repeat steps 313 to 315 at least twice until the buffer is clear of mucus.
317. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
318. Mince tissue with scissors.
319. Transfer tissue and buffer through sieve and discard flow through.
320. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
321. Shake tube by hand for 10 seconds.
322. Pour tissue and buffer through sieve and discard flow through.
323. Repeat steps 320 to 322 at least twice until the buffer is clear of mucus.
324. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
325. Mince tissue with scissors.
326. Transfer tissue and buffer through sieve and discard flow through.
327. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
328. Shake tube by hand for 10 seconds.
329. Pour tissue and buffer through sieve and discard flow through.
330. Repeat steps 327 to 329 at least twice until the buffer is clear of mucus.
331. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
332. Mince tissue with scissors.
333. Transfer tissue and buffer through sieve and discard flow through.
334. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
335. Shake tube by hand for 10 seconds.
336. Pour tissue and buffer through sieve and discard flow through.
337. Repeat steps 334 to 336 at least twice until the buffer is clear of mucus.
338. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
339. Mince tissue with scissors.
340. Transfer tissue and buffer through sieve and discard flow through.
341. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
342. Shake tube by hand for 10 seconds.
343. Pour tissue and buffer through sieve and discard flow through.
344. Repeat steps 341 to 343 at least twice until the buffer is clear of mucus.
345. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
346. Mince tissue with scissors.
347. Transfer tissue and buffer through sieve and discard flow through.
348. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
349. Shake tube by hand for 10 seconds.
350. Pour tissue and buffer through sieve and discard flow through.
351. Repeat steps 348 to 350 at least twice until the buffer is clear of mucus.
352. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
353. Mince tissue with scissors.
354. Transfer tissue and buffer through sieve and discard flow through.
355. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
356. Shake tube by hand for 10 seconds.
357. Pour tissue and buffer through sieve and discard flow through.
358. Repeat steps 355 to 357 at least twice until the buffer is clear of mucus.
359. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
360. Mince tissue with scissors.
361. Transfer tissue and buffer through sieve and discard flow through.
362. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
363. Shake tube by hand for 10 seconds.
364. Pour tissue and buffer through sieve and discard flow through.
365. Repeat steps 362 to 364 at least twice until the buffer is clear of mucus.
366. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
367. Mince tissue with scissors.
368. Transfer tissue and buffer through sieve and discard flow through.
369. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
370. Shake tube by hand for 10 seconds.
371. Pour tissue and buffer through sieve and discard flow through.
372. Repeat steps 369 to 371 at least twice until the buffer is clear of mucus.
373. Transfer tissue to 6 well plate containing 5ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
374. Mince tissue with scissors.
375. Transfer tissue and buffer through sieve and discard flow through.
376. Transfer tissue to 50ml tube containing 30ml RPMI/2% FBS/1% Pen-Strep/10mM HEPES/20mM NaHCO$_3$.
377. Shake tube by hand for 10 seconds.
378.

Acquisition of a multifunctional IgA+ plasma cell phenotype in the gut

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