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

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Method Article

Keywords: Flow Cytometry (FACS), Immunofluorescence Microscopy (IF), Mixed Bone Marrow Chimeras, Small intestinal lamina propria

Posted Date: December 16th, 2011

DOI: https://doi.org/10.1038/protex.2011.267

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+B220lowCD11cloiNOS+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+B220lowCD11cloiNOS+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.

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Acknowledgements

We thank Dionne White in the Faculty of Medicine Flow Cytometry core facility. C.P. is supported by a CIHR operating grant MOP# 9862. R.C. is supported in part by the Intramural Research Program of the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health. A.M. is supported by a CIHR operating grant MOP# 89783. J.H.F. acknowledges support by an APART-fellowship of the Austrian Academy of Sciences, McGill start-up funds and a CIHR operating grant MOP#114972. N.S. acknowledges the support of a CIHR Doctoral Award. J.L.G. is funded by the

Canadian Institutes of Health Research \(CIHR) and acknowledges the support of CIHR operating grant MOP# 67157 as well as infrastructure support from the Ontario Research Fund and that Canadian Foundation for Innovation.

Figures

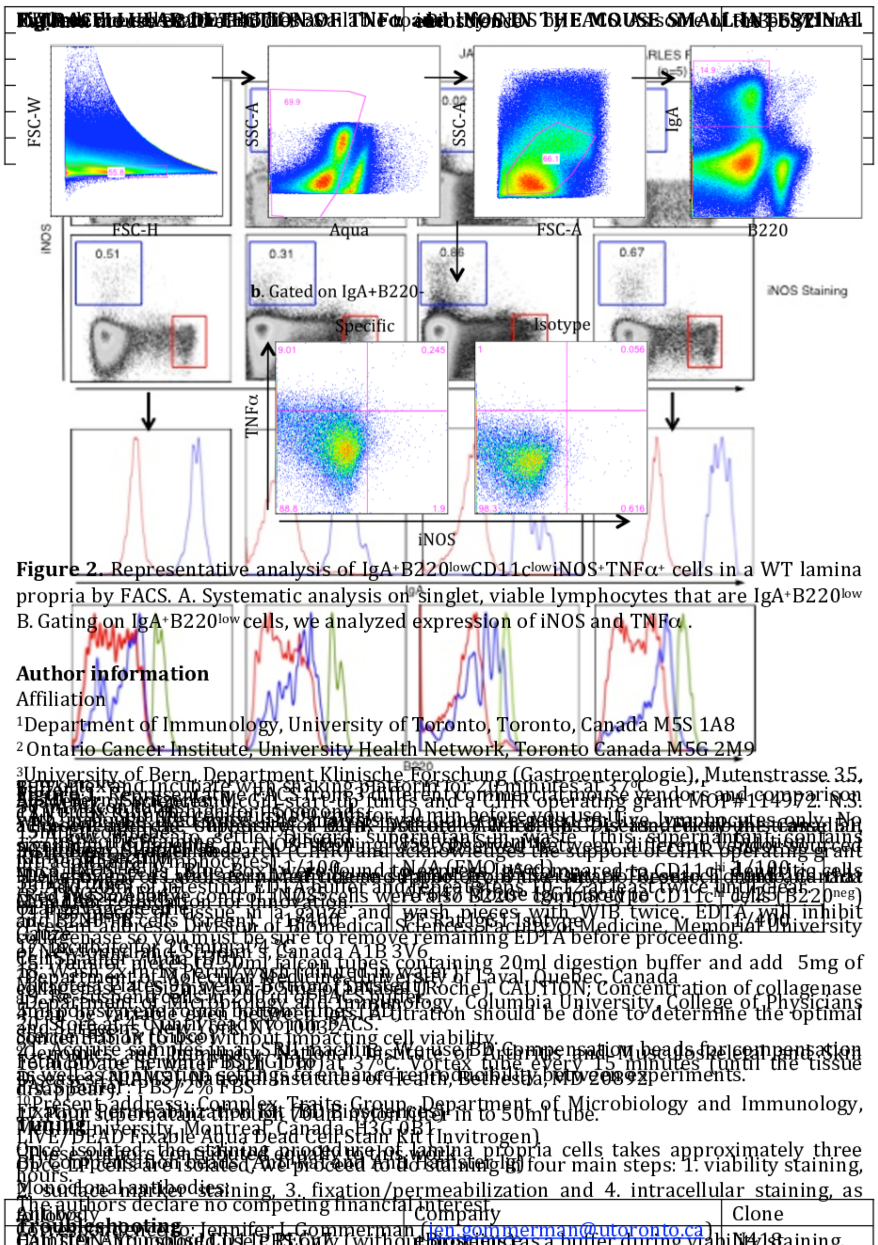

Figure 1

Complete Protocol with Figures 1-2 Detection of TNF/iNOS in small intestinal lamina propria cells