Detection of small intestinal IgA+TNFα+iNOS+ CELLS by Immunofluorescence Microscopy (IF)

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Method Article

Keywords: cryosections, microscopy, TNF, iNOS, Iga

Posted Date: December 16th, 2011

DOI: https://doi.org/10.1038/protex.2011.270

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) receptors 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. We report that IgA+ PC also can produce the anti-microbial mediators TNFα and iNOS, which appear to arise in the unique environment of the gut, and may be critical to mount effective responses to microbial assault. Here we described a detailed method to characterize and quantify IgA+iNOS+TNFα+ cells that we called TNFα-iNOS-producing \(Tip)-PC in the lamina propria of mice by immunohistochemistry \(IHC).

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) receptors 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. We report that IgA+ PC also can produce the anti-microbial mediators TNFα and iNOS, which appear to arise in the unique environment of the gut, and may be critical to mount effective responses to microbial assault. Here we described a detailed method to characterize and quantify IgA+iNOS+TNFα+ cells that we called TNFα-iNOS-producing \(Tip)-PC in the lamina propria of mice by immunohistochemistry \(IHC).

Reagents

Optimal Cutting Temperature \(OCT) compound \(Sakura Finetek) Base Molds \(15 x 15 x 5 mm, Fisher Scientific) 2-Methyl-Butane \(Fisher Scientific) Superfrost Plus Microscope Glass Slides and Coverslips \(Fisher Scientific) Tween-20 \(Sigma Aldrich) Tris-buffered saline \(TBS) TBS-T \(TBS plus 0.05% Tween-20) Phosphate buffered saline \(PBS) normal rabbit serum \(Jackson Laboratories) normal mouse serum \(Jackson Laboratories), bovine serum albumin \(BSA) \(Sigma) rat anti-mouse CD16/CD32 \(clone: 2.4G2, “Fc-block”) DAPI \(4',6-diamidino-2-phenylindole) nucleic acid stain \(InVitrogen) Gel/Mount \(Biomeda Corporation) Dako Pen \(Dako) Antibodies: 1. rat anti-mouse IgA-FITC, Southern Biotechnologies 11-44-2 2. rabbit anti-mouse iNOS-PE, Santa Cruz Biotechnology N20 3. rat anti-mouse CD8α-PE eBioscience 53-6.7 4. Anti-mouse monoclonal iNOS-ALEXA647 Santa Cruz Biotechnology C-11

5. rat anti-mouse TNFα-PE eBioscience MP6-XT22 6. rat anti-mouse TNFα-APC eBioscience MP6-XT22 7. rat anti-mouse EpCAM-APC eBioscience G8.8

Equipment

Leica CM3050 cryostat \(Leica Microsystems) Leica DMRA2 microscope \(Leica Microsystems) OpenLab imaging software \(Improvision) Retiga EXi digital camera \(Q Imaging) Photoshop CS5 \ (Adobe) Image J \(NIH)

Procedure

1. Cut the small intestines out of animals and remove the mesentery and fat. 2. After gently pushing out the fecal content using a forceps, wash the small intestines gently twice in PBS and cut open longitudinally and in small pieces of approximately 1 cm using a scissor. 3. Place tissue sections in base molds and cover with OCT compound and freeze in dry-ice-chilled 2-methyl-butane. 4. Wrap frozen OCT-tissue blocks in aluminum paper and store at -80°C until used for sectioning. 5. Cut intestinal tissue sections at 5 µm using a Leica CM3050 cryostat, mount on glass microscope slides, subsequently fix for seven minutes in ice-cold acetone and air-dry for one hour at room temperature. Fixed sections can be stored at -80°C in desiccant bags. 6. Using a Dako Pen, mount hydrophobic barrier rings onto glass slides by circling tissues to limit reagent use for staining. By doing so approximately 400 µl of liquid \(blocking or staining solutions) per slide is required. 7. Rehydrate sections in TBS for 20 minutes followed by additional 20 minutes in TBS-T by placing them in slide-beakers applying gentle shaking. 8. Block unspecific binding of antibodies by incubating tissue sections in TBS-T supplemented with 10% normal rabbit serum, 10% normal mouse serum, 5% sterile-filtered BSA and 2 mg/ml of a rat anti-mouse CD16/CD32 antibody for 30 minutes in a humidified chamber. After removal of the blocking solution, dip the slides briefly in TBS-T and then apply mixtures of the fluorochrome labeled antibodies diluted in TBS-T for 45 minutes in the dark in a humidified chamber. 9. After removal of the staining solutions, wash the slides three times for ten minutes in TBS-T, once with TBS and once with PBS in slide-beakers applying gentle shaking. 10. Stain the slides with DAPI nucleic acid stain for 30 seconds and wash three times with PBS in slide-beakers applying gentle shaking. 11. Mount the slides with Gel/Mount medium putting coverslips. 12. Acquire images with a Leica DMRA2 fluorescence microscope equipped with a Retiga EXi digital camera using OpenLab software. When comparing tissues of diverse genotypes, keep picture acquisition settings and exposure times for every fluorescent channel constant. 13. Process images utilizing Photoshop CS5. When comparing images of diverse genotypes or comparing specific antibody staining with isotype controls, adjustments of input and output levels was applied for all compared images in an equal manner. 14. Count the numbers of small intestinal LP IgA+ plasma cells and CD8α+ cells in a blinded fashion utilizing ImageJ. Analyse and quantify a total of five separate images from sections of six mice per group.

Timing

Harvest tissue and preparation of sections will require approximately four hours. The staining of tissue sections will then take approximately five hours.

Troubleshooting

1. We highly recommend to always apply a nucleic acid stain \(DAPI) or staining intestinal epithelial cell s \(EpCAM) as this will indicate you the quality of you tissue sections. Tissues depicting torn epithelial layers and/or disintegrated nuclei should be discarded. 2. As some stains might be dim, we recommend to immediately acquire pictures after staining tissues. Avoid light exposure of stained tissues at all times. 3. As staining intracellular antigens expressed at low levels can result in false positive results, we recommend to always include isotype control staining. If possible apply control staining utilizing tissues from TNFα and/or iNOS-deficient animals. 4. Spinning the intracellular cocktail before you use it helps to minimize the background.

Anticipated Results

Applying this staining methods we observes that IgA expression co-localizes with the expression of iNOS and TNFα with some, but not all IgA+ cells in the small intestinal LP tissue. Occasional co-expression of both iNOS and TNFα was observed in IgA+ cells, consistent with our flow cytometry data. Taken together, we have found that a proportion of IgA+ cells within the small intestinal LP express anti-microbial mediators TNFα and iNOS, while we do not detect significant expression of these molecules in other LP cells.

References

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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

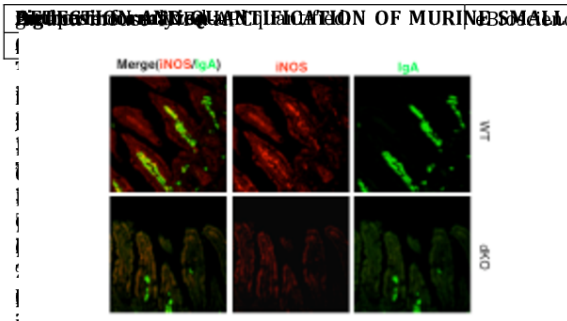

Figure 1

Detection of TNF/iNOS/IgA in the small intestinal lamina propria by IF