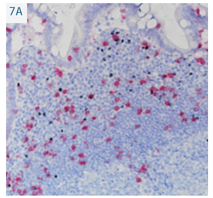
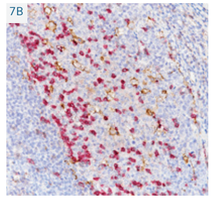
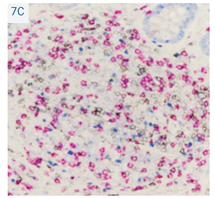
Figures 7: Small Intestine



FOXP3 (black) + CD8 (red)

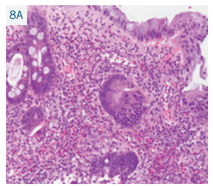


PD-1 (red) + PD-L1 (brown)

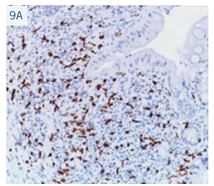


PD-1 (black) + CD8 (red) & FOXP3 (royal blue)

Figure 8 - 9: Inflammatory Bowel Disease



H&E



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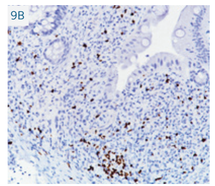

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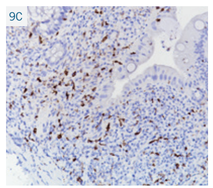

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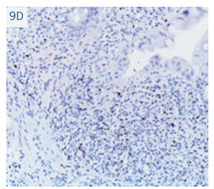
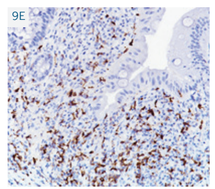
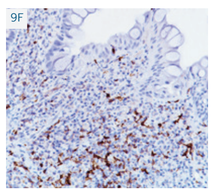


T-Bet



PD-1



OX40

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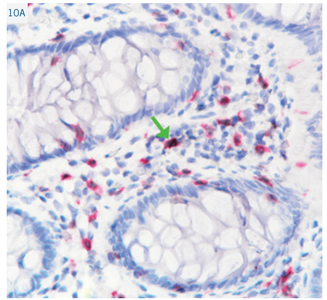
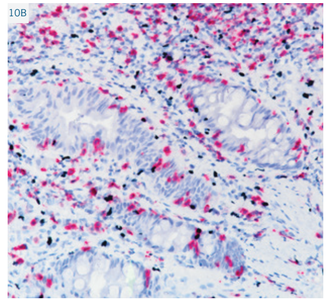
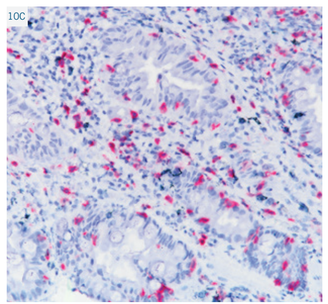
Figure 10: Inflammatory Bowel Disease



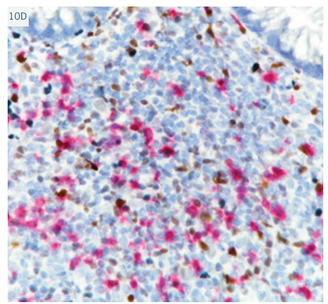
PD-1 (black) + CD8 (red). Notice co-expression of PD-1 + CD8 (green arrow)



OX40 (black) + CD8 (red)



FOXP3 (black) + CD8 (red)



FOXP3 (black) + CD8 (red) and RORγT (brown)

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Deciphering the Immune System in Small Intestine: Immune Related Antibodies and Multiplex Strategies for Inflammatory Bowel Disease

David Altree-Tacha, PhD, Wei Yuan, PhD, George Yang, MD. Biocare Medical, Pacheco, CA; USA

Introduction

Inflammatory bowel disease (IBD) can affect the lower part of the small intestine (ileum) and the colon.[1,2] When defining IBD, the typical clinical manifestations are Crohn's disease or ulcerative colitis.[2] Patients with IBD involving the colon may be at increased risk for developing colorectal cancer (CRC).[3] Although CRC caused from ulcerative colitis and Crohn's disease only accounts for 1 to 2% of all cases of CRC, however, IBD is considered a serious complication of the disease and accounts for approximately 15% of all deaths in patients with IBD.[4] Recent IBD studies have shown great promise for potential therapeutic treatments.[5-8] Evidence suggests that several factors may tip the balance between homeostasis and intestinal inflammation, and thus, present future challenges for the development of tools to improve diagnosis, provide a more accurate prognosis, and potential immunotherapies for IBD.[5-8]

In this study, we use normal small intestine (SI) as a model to identify multiple immune targets using monoclonal mouse and rabbit antibodies. Peyer's patches (PP) are lymphoid tissues in the wall of the SI. PP are involved in the development of immunity to antigens presented in the surrounding areas.[9] Morphological locations of immune interest include: the germinal center (GC), the mantle zone (MTZ), which is located on the outer ring of GC; the marginal zone (MZ), located outside the MTZ and adjacent to non-lymphoid tissue; and intestinal villi.[10,11] SI and cases of IBD were evaluated as potential models using immunohistochemistry (IHC) to identify categories of immune cells including cytotoxic T-cells, costimulatory cells (activators), macrophages, T-regulatory cells (TREGs), and to examine expression patterns of immune checkpoint proteins. Cases of SI and IBD were examined via IHC single and multiplex stains.

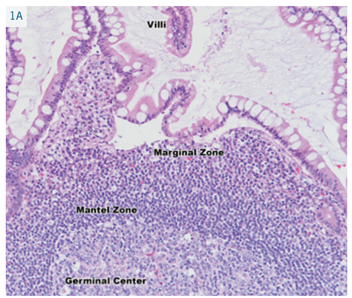
Design

Formalin-fixed paraffin-embedded human cases of SI and IBD were cut at 4 microns and stained with hematoxylin and eosin (H&E). Mouse and rabbit monoclonal antibodies including CD8, CD163, CD103, CD137, OX40, FOXP3, LAG3, GATA3, T-bet, RORγT, PD-1 and PD-L1 (Biocare Medical) were titered for staining optimization and evaluated by IHC. Antibodies were detected with a one-step polymer detection system and visualized with DAB chromogen.

The staining pattern of each antibody was categorized according to cell type, cell function, cell location, and morphological location in SI. IHC multiplex stains were also evaluated on SI, including PD-1 + CD8, FOXP3 + CD8, and PD-1 + CD8 & FOXP3. All cocktails were applied to tissue sections, followed by a double or triple stain polymer detection system; and visualization with Warp Fast Red, Deep Space Black, Ferangi Blue and Betazoid DAB (brown) chromogens (Biocare Medical).

Additionally, several cases of IBD were selected for single and multiplex stains including PD-1 + CD8, FOXP3 + CD8, OX40 + CD8 and a triple stain with FOXP3 + CD8 & RORγT antibodies.

Figure 1



Peyer patch (H&E) staining zones: germinal center, mantle zone and marginal zone

As Presented at AACR 2017, Poster Session #3817

Results

An H&E section of a Peyer patch SI with defined morphological zones demonstrated GC, MTZ, MZ and intestinal villi (Figure 1). Antibodies tested were defined by cell type, cell function, cellular location and morphological location in SI (See Table 1).

Small Intestine

Cellular Location (Table 1)

The IHC cellular location of each antibody tested included membrane/cytoplasm, cytoplasmic, nuclei and cell membranes. Cytotoxic T-cells were cell membrane/cytoplasm; macrophages demonstrated cytoplasmic staining; we observed nuclear staining in TREGs with the exception of LAG3 (cell membrane), and immune checkpoint inhibitors demonstrated cell membrane staining.

Morphological Locations: (Table 1)

Cytotoxic T-cells showed low staining in GC and moderate to strong staining in MTZ, MZ and villi (Figure 2). CD163 (macrophages) showed moderate to strong staining in all zones (Figure 3). OX40 (checkpoint activator) showed low to moderate staining in GC and MZ (Figure 4A), and CD137 (checkpoint activator) showed the strongest staining (follicular dendritic cells) in GC (Figure 4B). CD103 (T-cell activator) demonstrated no expression in GC and MTZ, and moderate expression in the MZ and villi (Figure 4C). TREGs (FOXP3, RORγT and T-bet) showed virtually no expression in GC and MTZ (Figure 5 A-C), except for GATA3 (Figure 5D), which demonstrated robust staining in all zones, while LAG3 (cell membrane staining) was expressed mainly in GC (Figure 5E). Immune checkpoint markers (PD-1 and PD-L1) demonstrated moderate to strong staining in GC (Figure 6A, B). Double and triple stains were easily adapted in SI and showed equivalent staining to single stains (Figure 7: A-C).

Inflammatory Bowel Disease

IBD cases were confirmed with H&E (Figure 8). Single IHC stained cases of IBD used identical titers obtained from SI. Strong and robust staining was observed with all antibodies tested (Figures 9: A-F). All double and triple stains showed discrete staining with each antibody being easily distinguishable (Figure 10: A-D).

Conclusion

Small intestine is a good model to develop IHC for a host of immune cell targets. Enhanced visualization of these targets is possible with the use of double and triple stains, and thus may help facilitate acquisition of important diagnostic and prognostic information. We posit these approaches may also support new therapeutic strategies for inflammatory bowel disease by leveraging immunotherapeutic agents.

References

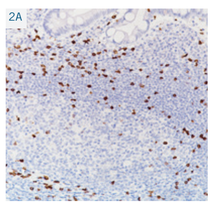
1. Yi-Zhen Zhang, *et al.* Inflammatory bowel disease: pathogenesis. World J Gastroenterol. 2014 Jan 7;20(1):91-9. 2. Manabe T, *et al.* Feasibility of laparoscopic surgery for complex Crohn's disease of the small intestine. Asian J Endosc Surg. 2016 Nov;9(4):265-269. 3. Yu JX, *et al.* Surveillance of patients with inflammatory bowel disease. Best Pract Res Clin Gastroenterol. 2016 Dec;30(6):949-958. 4. Munkholm P. Review article: the incidence and prevalence of colorectal cancer in inflammatory bowel disease. Aliment Pharmacol Ther. 2003 Sep;18 Suppl 2:1-5. 5. Li J, *et al.* Profiles of Lamina Propria T Helper Cell Subsets Discriminate Between Ulcerative Colitis and Crohn's Disease. Inflamm Bowel Dis. 2016 Aug;22(8):1779-92. 6. Tom MR, *et al.* Novel CD8+ T-Cell Subsets Demonstrating Plasticity in Patients with Inflammatory Bowel Disease. Inflamm Bowel Dis. 2016 Jul;22(7):1596-608. 7. Carbonnel F, *et al.* Inflammatory bowel disease and cancer response due to anti-CTLA-4: is it in the flora? Semin Immunopathol. 2017 Jan 16. 8. Zamani MR, *et al.* PD-1/PD-L and autoimmunity: A growing relationship. Cell Immunol. 2016 Dec;310:27-41. 9. Jung C, *et al.* Peyer's Patches: The Immune Sensors of the Intestine. Int J Inflam. 2010 Sep 19;2010:823710. 10. http://teaching.path.cam.ac.uk/partIB_pract/P05/ 11. Richard J. Bende, *et al.* Chronic inflammatory disease, lymphoid tissue neogenesis and extranodal marginal zone B-cell lymphomas Haematologica August 2009 94: 1109-1123.

Table 1

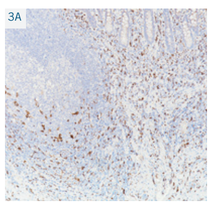
Antibody	Cell Type	Cell Function	Cellular Location	Germinal Center	Mantle Zone	Marginal Zone	Intestinal Villi
CD8	Cytotoxic T cell	Killing target cells	Membrane/cytoplasm	Low expression	High expression	High expression	Moderate expression
CD163	Macrophage	Engulfing and destroying target cells	Membrane/cytoplasm	Moderate expression	Moderate expression	High expression	High expression
CD103	Integrin mediating lymphocyte	Activation of cytotoxic T cells	Membrane/cytoplasm	No expression	Trace expression	Moderate expression	Moderate expression
OX40 (CD134)	Activated T cell/natural killer cell	Checkpoint co-stimulatory for effector T cells	Membrane/cytoplasm	Moderate expression	Trace expression	Moderate expression	Low expression
CD137 (4-1BB)	Follicular dendritic cells and T cell	Checkpoint co-stimulatory for effector T cells	Cell membrane	High expression	Low expression	Low expression	Trace expression
FOXP3	T-regulatory cell	Turns the immune response down (brake)	Nuclei	No expression	Low expression	Moderate expression	Low expression
LAG3	T-regulatory cell	Turns the immune response down (brake)	Cell membrane	Low expression	Trace expression	Low expression	Low expression
GATA3	T-regulatory cell (Th2)	Downregulates inflammatory responses	Nuclei	Moderate expression	High expression	High expression	Moderate expression
T-bet	T-regulatory cell (Th2)	Downregulates inflammatory responses	Nuclei	No expression	Low expression	High expression	Moderate expression
ROR gamma T	T-helper cell (Th17)	Pro-inflammatory function (IL-17)	Nuclei	Low expression	High expression	High expression	Moderate expression
PD-1	T cell, natural killer cell, B cell	Down regulates T cells (stop mechanism)	Cell membrane	High expression	Moderate expression	High expression	Low expression
PD-L1	Tumor cell and immune cell	Modulate activation/inhibition of T or B cells	Cell membrane	Moderate	Low expression	Trace expression	Trace expression

IHC expression was evaluated under light microscopy.

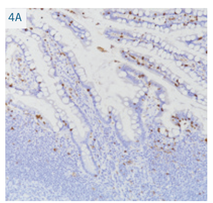
Figures 2-6: Small Intestine



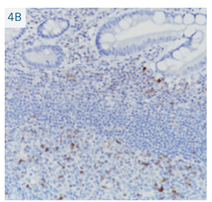
CD8 (cytotoxic T-cell)



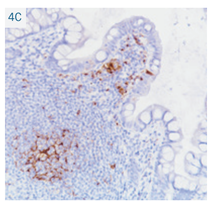
CD163 (macrophage)



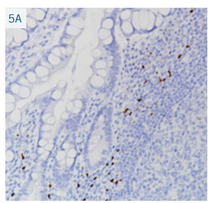
CD103 (activator)



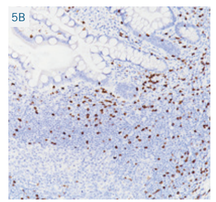
OX40 (checkpoint activator)



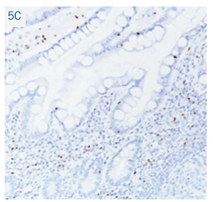
CD137 (checkpoint activator)



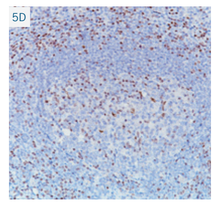
FOXP3 (TREG)



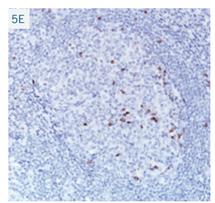
RORγT (TREG Th17)



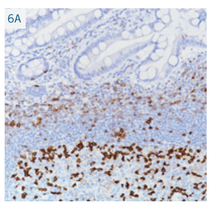
T-Bet (TREG Th2)



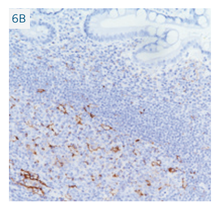
GATA3 (TREG Th2)



LAG3 (TREG)



PD-1 (Immune checkpoint)



PD-L1 (Immune checkpoint)

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