APPLICATION NOTE

VISUALIZATION OF LGR5+ STEM CELLS AND THE IMMUNE RESPONSE IN THE INFLAMED MOUSE COLON BY THE RNAscope® *IN SITU* HYBRIDIZATION ASSAY

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HIGHLIGHTS

Targets examined in this study include:

- Intestinal stem cell markers
- Wnt/β-catenin pathway molecules
- Immune cell markers
- Cytokines and chemokines

Detection of these RNA targets with the RNAscope® assay can aid in:

- Elucidating direct effects of inflammatory cues on the ISCs and their niche during inflammation
- Developing potential therapeutics for intestinal bowel disease (IBD) and other inflammatory diseases
- Visualizing relationships between ISCs and other cell types in the intestinal crypt
- Identifying specific immune cell types in the inflamed colon
- Characterizing secreted proteins (i.e. cytokines and chemokines)

MD + MC

MOLECULAR DETECTION + MORPHOLOGICAL CONTEXT IN A SINGLE ASSAY

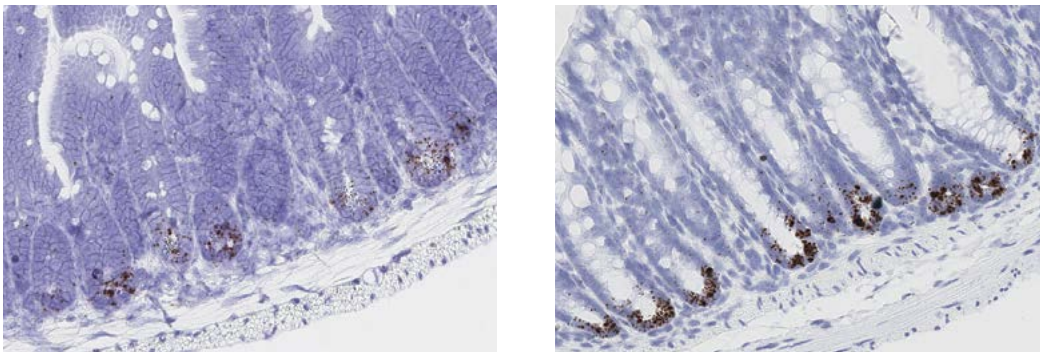
INTRODUCTION

The RNAscope® assay is a unique RNA ISH technology that identifies RNA expression at the single cell level with morphological context. Here we present the use of the RNAscope® assay to visualize intestinal stem cells (ISCs) within the morphological context of the intestinal crypt and in relationship to inflammatory immune cells.

Due to its exposure to a harsh luminal environment, the epithelium of the adult mammalian intestine has a remarkably fast turnover rate that is facilitated by a resident intestinal stem cell (ISC) population present at the base of the intestinal crypt. These ISCs, marked by the Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5), allow the intestinal epithelium to adapt to different ztypes of damage, such as inflammation[1, 2]. Chronic intestinal inflammation is a hallmark of the inflammatory bowel diseases (IBD), and intestinal organoids generated from adult ISCs are a promising treatment for IBD, yet the interplay between inflammatory immune cells and ISCs remains to be elucidated[2]. The intestinal crypt is a well-defined structure in cellular orientation and differentiation, making it an ideal model system to study the cellular responses of immune and stem cells during inflammation.

RNAscope® is a unique RNA ISH technology that provides single-cell gene expression resolution with spatial and morphological context. The RNAscope® assay detects mRNA and long non-coding RNAs in fresh frozen, fresh fixed, and formalin-fixed paraffin-embedded (FFPE) cells and tissues by utilizing a unique double Z probe design and signal amplification strategy that allows for visualization of target RNA as a single dot, where each dot is an individual RNA molecule[3]. The key benefit of the RNAscope® technology is high sensitivity due to its amplification method and high specificity because of the double Z probe design, leading to a high signal-to-noise ratio in many tissues.

This assay is a well-suited method for the cellular resolution of resident ISCs and their progenitors in the normal and inflamed intestine because it detects single RNA molecules in individual cellswith morphological context.



Visualization of the *Lgr5* intestinal stem cell population in the small (left) and large (right) mouse intestine with the RNAscope® assay.

RESULTS

In this report, we utilized the RNAscope® assay to visualize the expression of multiple immune and ISC markers within the inflamed intestinal tissue environment (summarized in TABLE 1). To interrogate the expression pattern of inflammatory immune cell and ISC markers within the intestinal crypt, we performed the assay on colons from either control or TNBS-treated mice. We visualized the location of the resident Lgr5+ ISC population within the colonic crypt (**FIGURES 1 & 4**), as well as other cell populations in the small intestine (**FIGURES 2 & 3**). The impact of inflammation on the Lgr5+ ISC population, as well as the Wnt/β-catenin pathway, was also examined (**FIGURES 4 & 5; APPENDIX**). Using the RNAscope® Multiplex Fluorescent assay, we assessed the presence of immune cells in the inflamed region (**FIGURES 6 & 7**). Lastly, we examined the expression of several receptor-ligand pairs for cytokines and ISC markers (**FIGURE 8**). The complete data set, including the appendix, is available online at www.acdbio.com/stemcells.

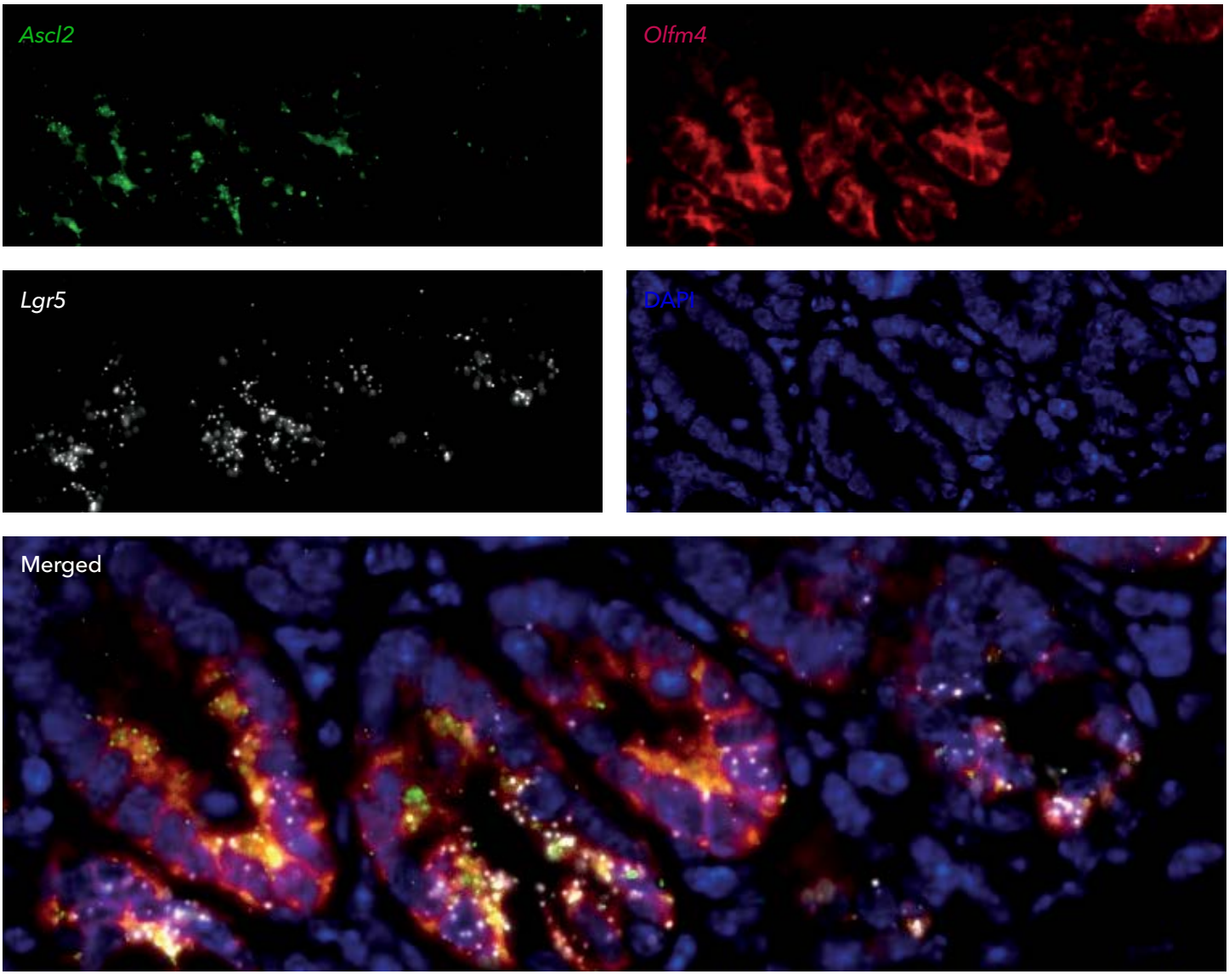


FIGURE 1. Visualization of intestinal stem cell (ISCs) in the adult mouse small intestine. The RNAscope® Multiplex Fluorescent assay was used to simultaneously detect three markers of the ISC population localized to the crypt base of the adult mouse small intestine: *Ascl2* (green), *Olfm4* (red), and *Lgr5* (white); nuclei were stained using DAPI (blue).

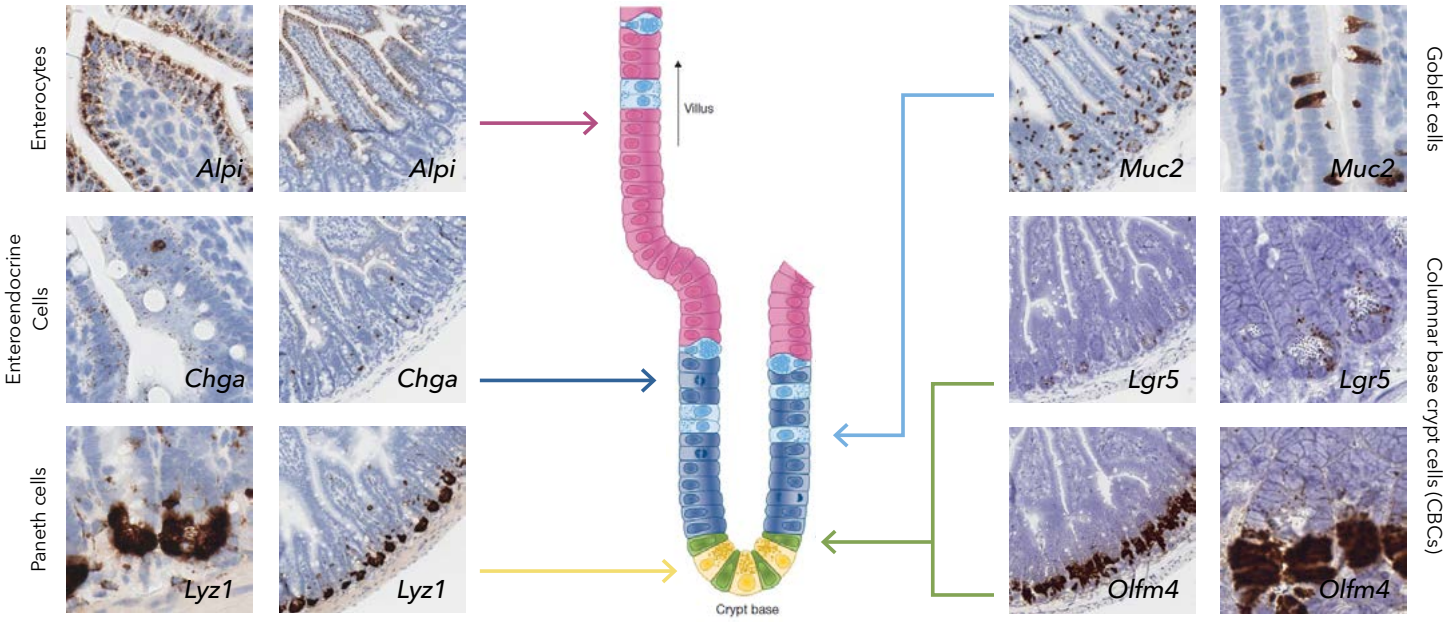


FIGURE 2. Identification of distinct intestinal cell populations. Five intestinal cell populations were identified by the RNAscope® assay. Enterocytes were marked by *Alpi*, enteroendocrine cells were marked by *Chga*, Goblet cells were marked by *Muc2*, Paneth cells were marked by *Lyz1*, and columnar base crypt cells (CBCs) were marked by *Lgr5* and *Olfm4*.

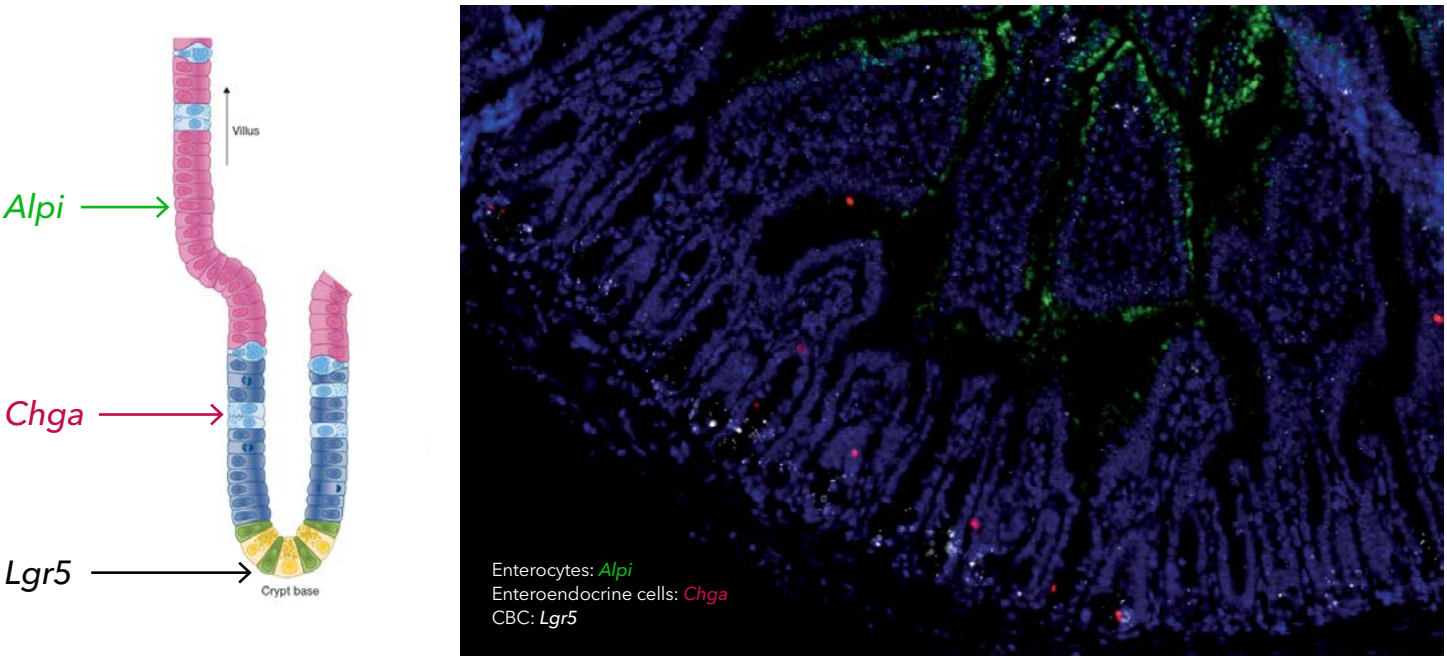


FIGURE 3. Simultaneous detection of three intestinal cell populations. Enterocytes were marked by *Alpi* (green), enteroendocrine cells were marked by *Chga* (red), and columnar base crypt cells (CBCs) were marked by *Lgr5 (white)* using the RNAscope® Multiplex Fluorescent assay. Nuclei were stained with DAPI (blue).

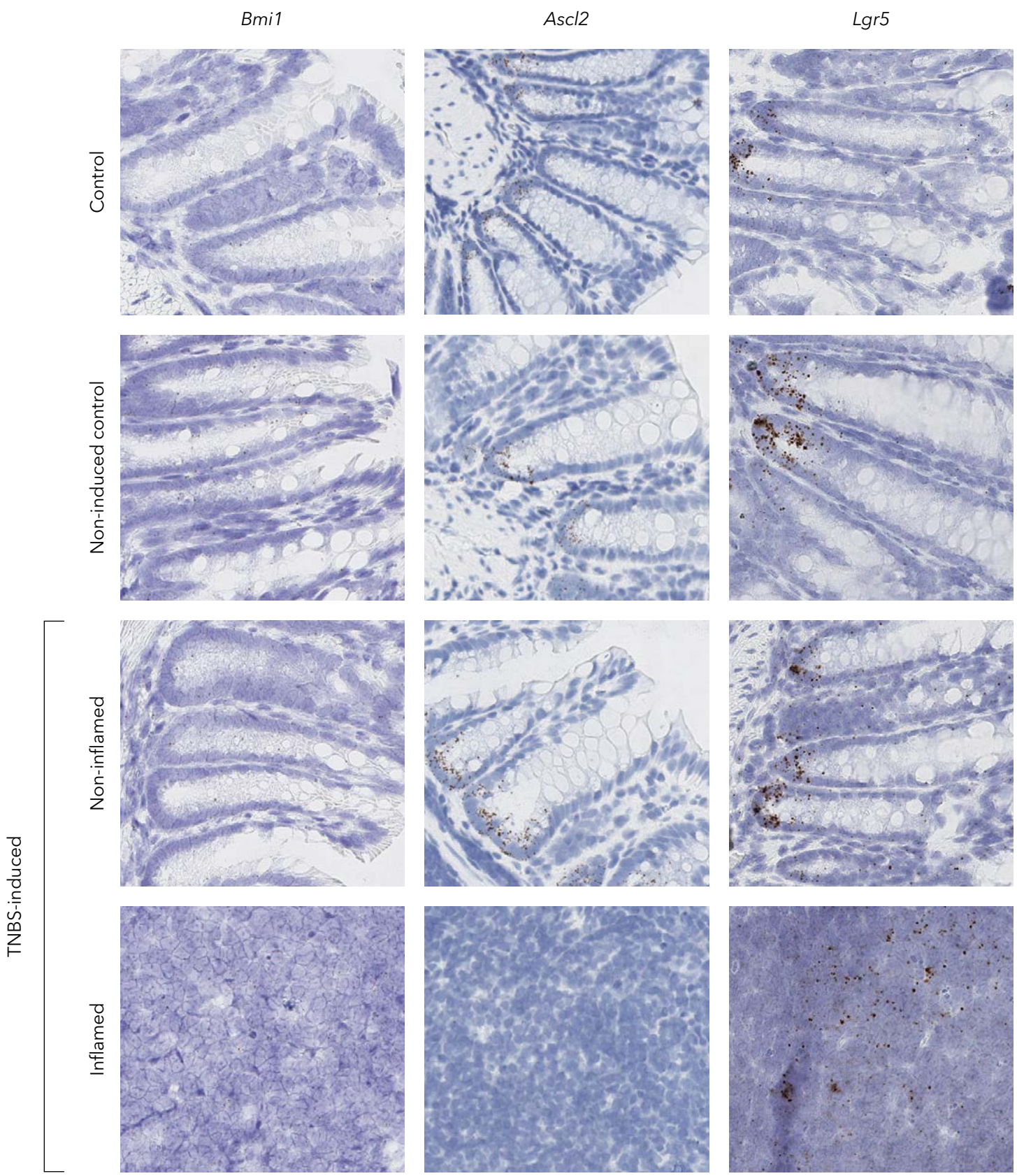


FIGURE 4. Visualization of intestinal stem cell (ISCs) in the normal and inflamed adult mouse colon using the RNAscope® assay. *Lgr5* and *Ascl2* expression was localized primarily to the base of the crypt, while *Bmi1* was expressed throughout the crypt. However, in the inflamed region, *Lgr5* was strongly expressed, while little to no expression of *Ascl2* and *Bmi1* was detected.

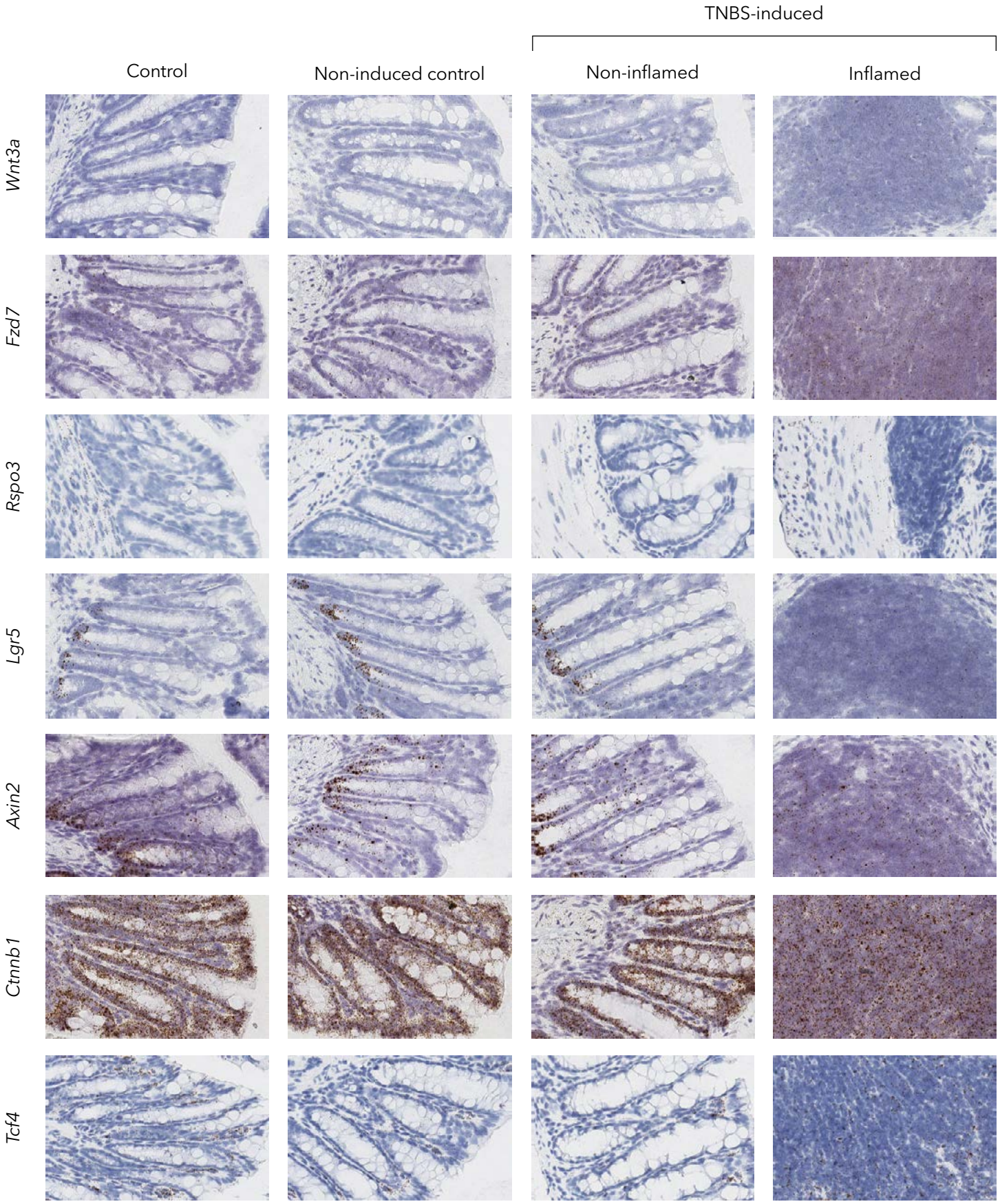


FIGURE 5. Visualization of the Wnt/β-Catenin pathway in the normal and inflamed adult mouse colon. The RNAscope® assay was used to detect expression of several components of the Wnt/β-Catenin pathway in both the normal and inflamed adult mouse colon.

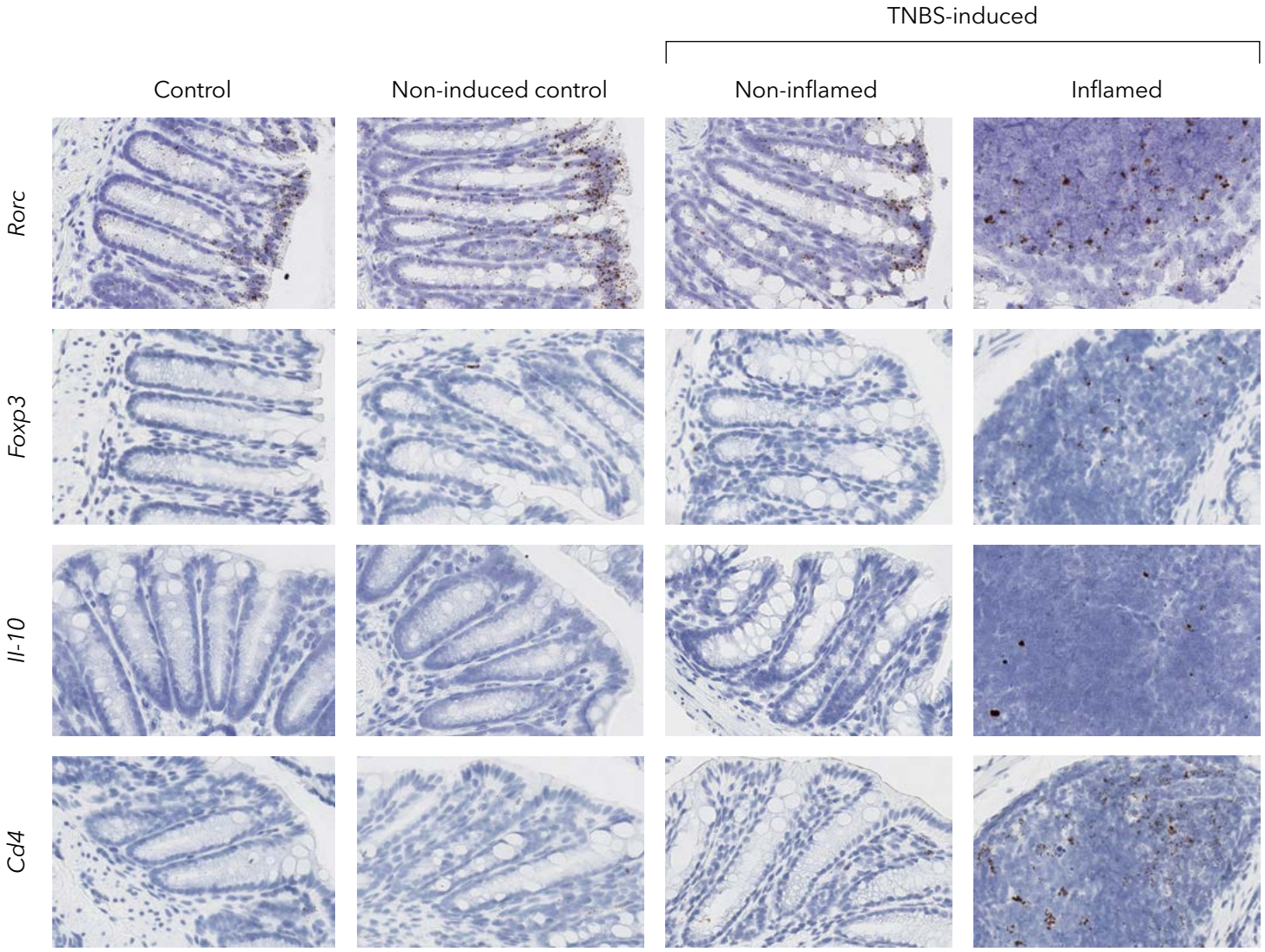
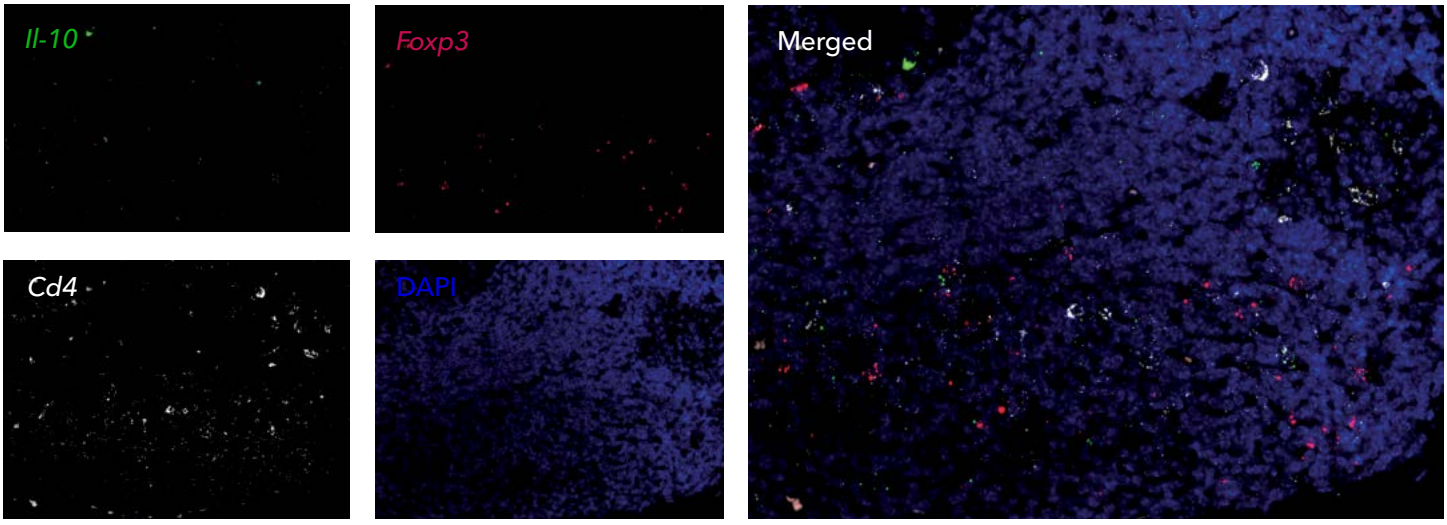


FIGURE 6. Detection of the immune cell response during inflammation of the adult mouse colon. The RNAscope® assay was used to visualize the expression of the immune cell markers *Rorc*, *Foxp3*, *Il-10*, and *Cd4* in the normal and inflamed adult mouse colon.

A



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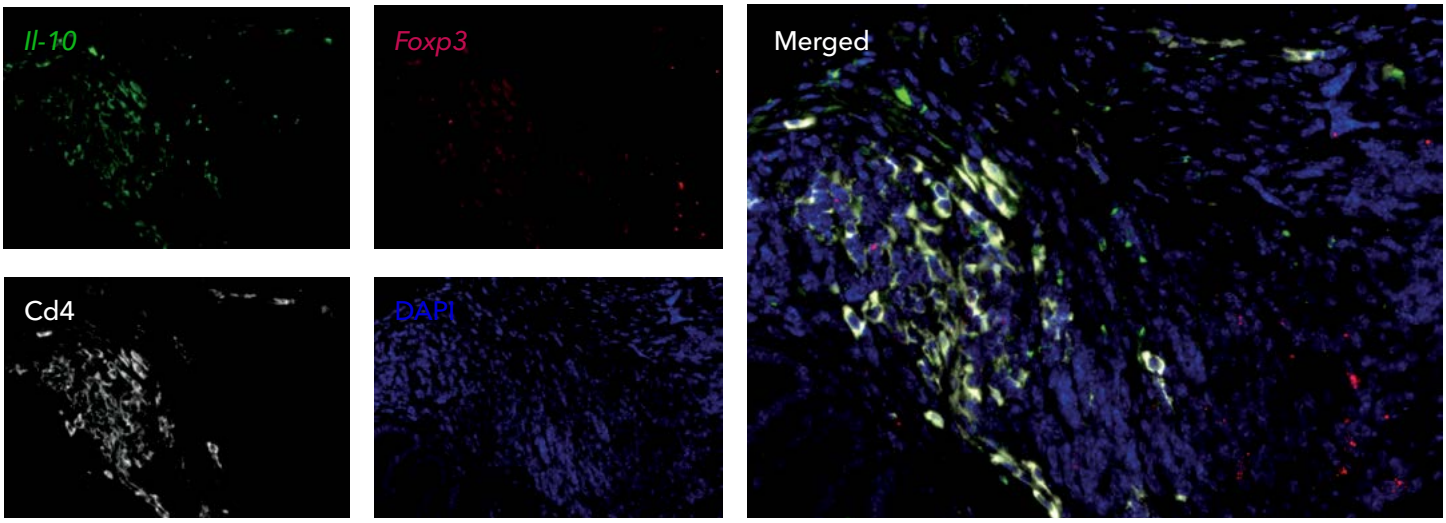


FIGURE 7. Detection of regulatory T cells in the inflamed adult mouse colon. (**A**) The RNAscope® Multiplex Fluorescent assay was used to simultaneously detect three markers of the regulatory T cell (Tregs) population in the inflamed colon: *Il-10* (green), *Foxp3* (red), and *Cd4* (white). (**B**) The RNAscope® Multiplex ISH-IHC Fluorescent assay was used to simultaneously detect *Il-10* (green) and *Foxp3* (red) mRNA and Cd4 protein (white) in the inflamed colon. Nuclei were stained with DAPI (blue).

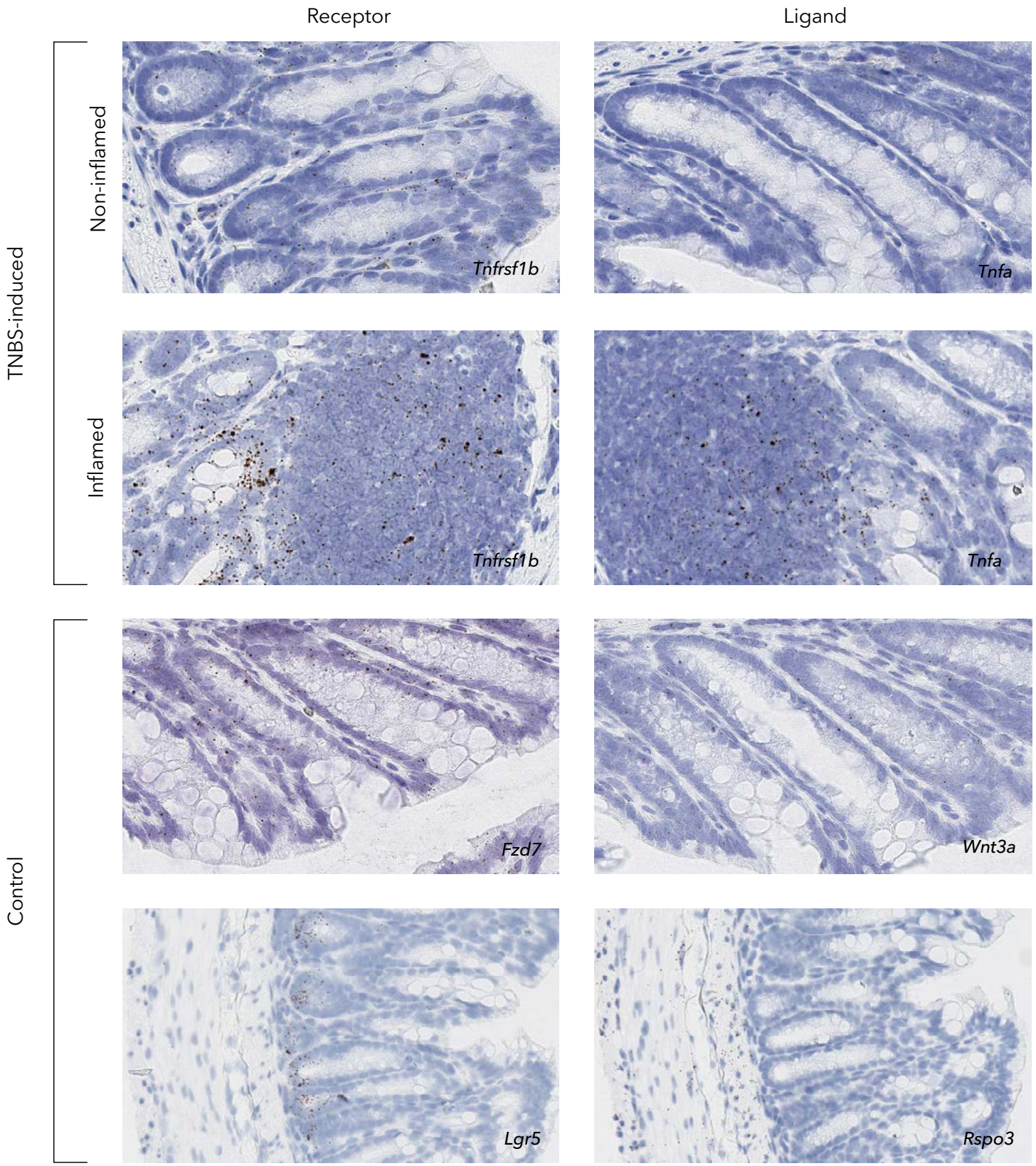


FIGURE 8. Visualization of receptor-ligand partners in the adult mouse colon. Several receptor-ligand partners were detected using the RNAscope® assay in both the normal and inflamed adult mouse colon, including *Tnfα* and its receptor *Tnfrsf1b*, *Wnt* and its receptor ***Fzd***, and Rspo and its receptor *Lgr5*.

CONCLUSIONS

Detecting RNA biomarker expression at the single-cell level while preserving spatial information is critical to understanding cellular organization and cell-to-cell interactions during ISC proliferation and differentiation as well as intestinal inflammation. Here we show that the RNAscope® assay is able to visualize the ISCs within the morphological context of the intestinal crypt and in relationship to inflammatory immune cells. Overall these results demonstrate the utility of the RNAscope® technology in elucidating the direct effects of inflammatory cues on the ISCs and their niche during the pathogenesis of IBD and other inflammatory diseases, as well as developing potential therapeutics.

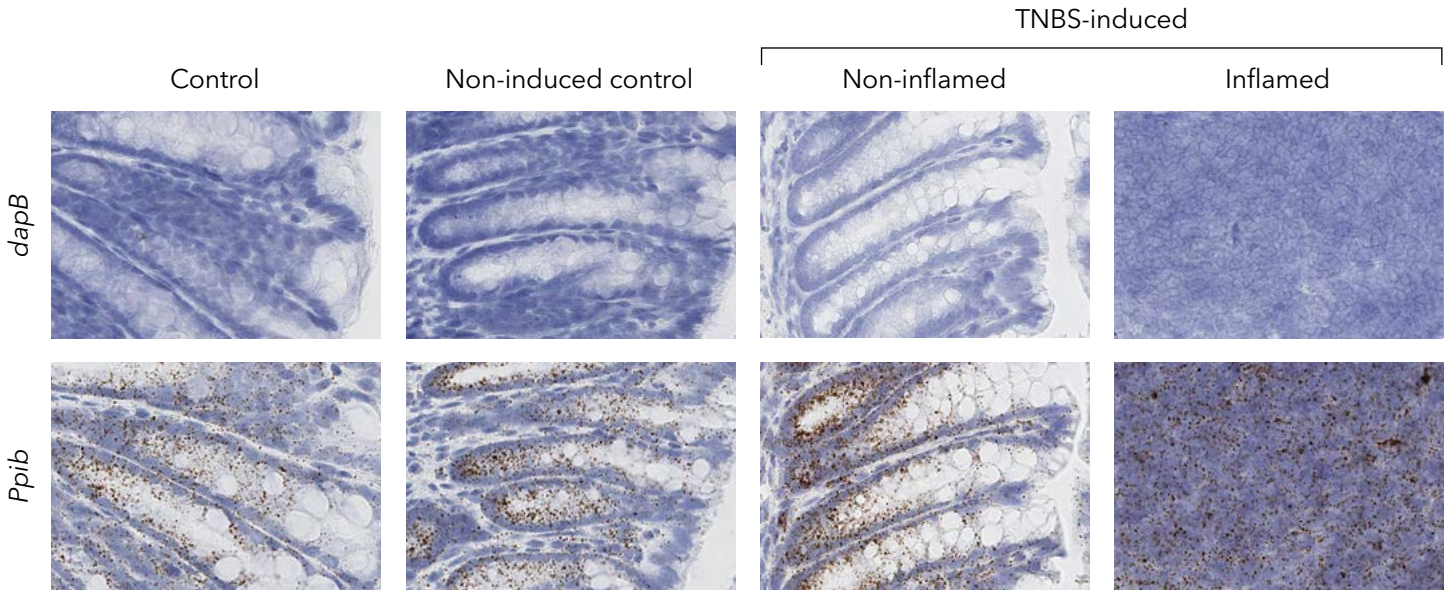
REFERENCES

1. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature*. 2007; 449(7165):1003-7.
2. Barker N. Adult intestinal stem cells: Critical drivers of epilthelial homeostasis and regeneration. Nat Rev *Mol Cell Biol*. 2014. 15(1):19-33.
3. Wang F, Flanagan J, Su N, Wang LC, Bui S, Nielson A, Wu X, Vo HT, Ma XJ, Luo Y. RNAscope: A novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J Mol Diagn*. 2012; 14(1):22-9.

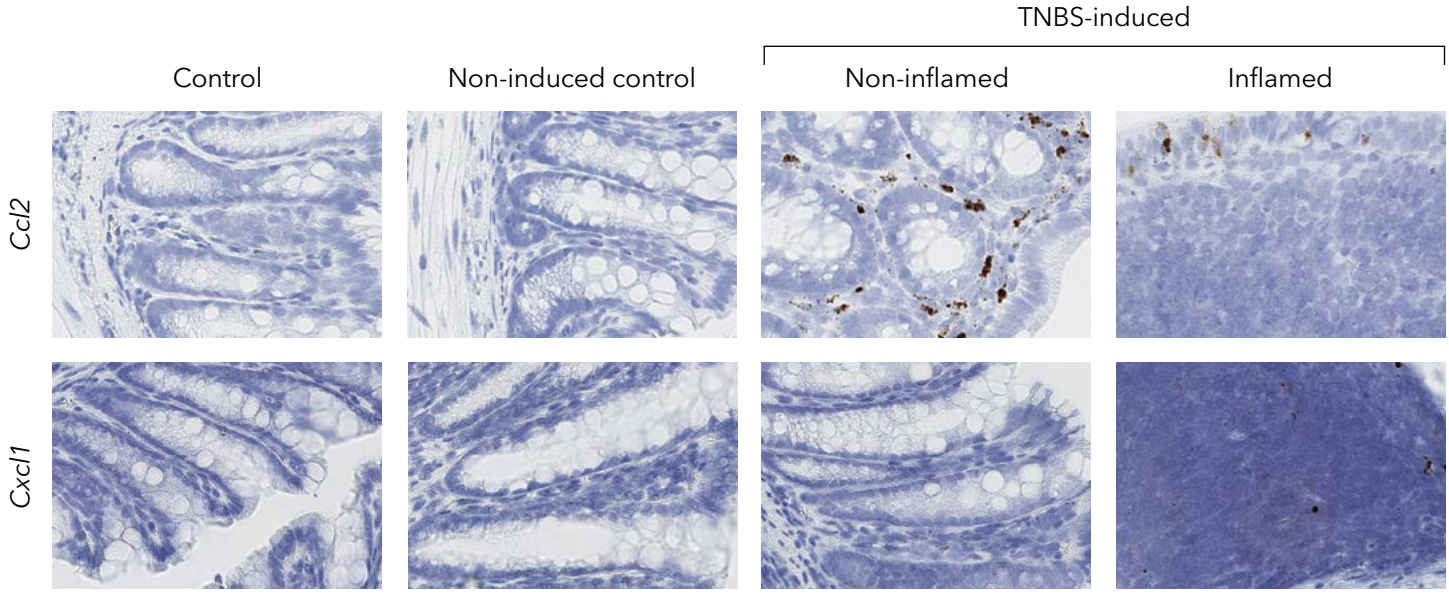
MARKERS	PROBE NAME	CATALOG NUMBER
INTESTINAL AND STEM CELL-RELATED MARKERS		
Alpi	Mm-Alpi	436781
Ascl2	Mm-Ascl2	412211
Axin2	Mm-Axin2	400331
Bmi1	Mm-Bmi1-O1	466021
-catenin (Ctnnb1)	Mm-Ctnnb1	311741
Chga	Mm-Chga	447851
Fzd7	Mm-Fzd7	404931
Lgr5	Mm-Lgr5	312171
Lyz1	Mm-Lyz1	415131
Muc2	Mm-Muc2	315451
Olfm4	Mm-Olfm4	311831
Rspo3	Mm-Rspo3	402011
Tcf4	Mm-Tcf4	423691
Wnt3a	Mm-Wnt3a	405041
IMMUNE CELL MARKERS		
Cd4	Mm-Cd4	406841
Foxp3	Mm-Foxp3	432611
RORγ (Rorc)	Mm-Rorc	403661
CYTOKINES AND CHEMOKINES		
Ccl2	Mm-Ccl2	311791
Csf2	Mm-Csf2	319701
Cxcl1	Mm-Cxcl1	407721
Il-1β (Il1b)	Mm-Il1b	316891
Il-5	Mm-Il5	319381
Il-6	Mm-Il6	315891
Il-8 (Cxcl15)	Mm-Cxcl15	409101
Il-10	Mm-Il10	317261
Il-12β (Il12b)	Mm-Il12b	319551
Tnf-α (Tnfa)	Mm-Tnfa	311081
Tnfrsf1b	Mm-Tnfrsf1b	435941

TABLE 1. Intestinal stem and immune cell markers examined in this study using the RNAscope® assay. In this study, we examined 28 markers, including 14 intestinal and stem cell-related markers, 3 immune cell markers, and 14 cytokines and chemokines.

APPENDIX

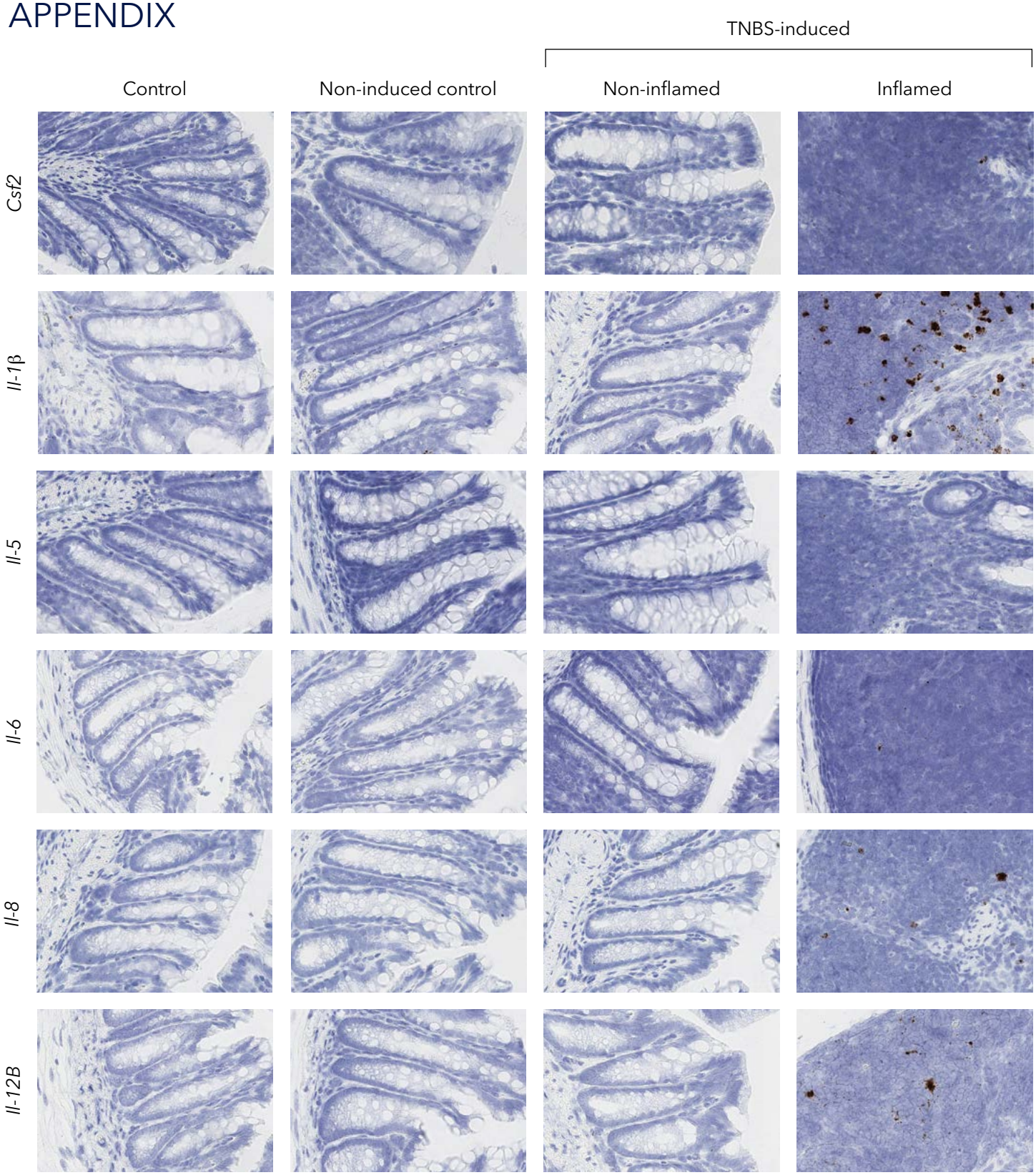


APPENDIX FIGURE 1. Quality control of the normal and inflamed adult mouse colon. RNA quality of all the tissues examined in this study was confirmed using the RNAscope® assay with control probes. No signal was observed with the negative control probe *dapB* while strong, uniform signal was observed with *Ppib*.



APPENDIX FIGURE 2. Additional chemokines examined in the normal and inflamed adult mouse colon. The RNAscope® assay was used to visualize the expression of the chemokines *Ccl2* and *Cxcl1* in the normal and inflamed adult mouse colon.

APPENDIX



APPENDIX FIGURE 3. Additional cytokines examined in the normal and inflamed adult mouse colon. The RNAscope® assay was used to visualize the expression of the cytokines *Csf2, Il-1β, Il-5, Il-6, Il-8,* and *Il-12B* in the normal and inflamed adult mouse colon.

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