UNIVERSITY OF CALIFORNIA, SAN DIEGO

Mechanisms Underlying Diarrheal Disease in Enteric *Salmonella* Infections

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

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2017

The Thesis of Rashini Jayaratne is approved, and is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2017

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SUPPLEMENTARY FIGURES/TABLES

ABSTRACT OF THE THESIS

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Non-typhoidal *Salmonella* is the most burdensome foodborne illness worldwide, yet despite its prevalence, the mechanism through which *Salmonella* causes diarrhea is not entirely known. Intestinal ion transporters play important roles in water absorption in the intestine, and can be a therapeutic target in *Salmonella* induced diarrhea, but there is not a complete understanding of which ion transporters *Salmonella* regulates. We have previously shown that infection with *Salmonella* causes decreased expression

of the chloride/bicarbonate exchanger SLC26A3 (DRA; Down-Regulated in Adenoma) in the colonic tissues of mice. To better understand the mechanism of *Salmonella* infection in intestinal epithelial cells, we developed an enteroid model that mimics the crypt-villus axis of the intestines, and contains the four different cell subtypes found in the intestines (enterocytes, enteroendocrine, goblet, and Paneth cells). These enteroids were converted to enteroid-derived monolayers (EDM) to allow access to the apical membrane. With this model, we looked at changes in the Wnt/NOTCH signaling pathways responsible for controlling cell fate commitment in the intestines in addition to changes in DRA in both colonic and small intestinal EDM. We hypothesized that EDM would show similar decreases in DRA to those seen *in* vivo, in addition to alterations in Wnt/NOTCH signaling that may be linked to DRA down-regulation. We found that expression of both DRA and NICD (of the NOTCH signaling pathway) were decreased 6 h post infection. Our work shows the possibility of using EDM as a model to uncover mechanisms that can be targeted for therapeutic developments against diarrhea caused by *Salmonella* infection.

INTRODUCTION

The Pathophysiology of Salmonella Infections

Non-typhoidal *Salmonella* spp. *(*e.g., *Salmonella* enterica serovar *typhimurium)* are the most burdensome food-borne diarrheal pathogens; of the approximate 2.8 billion cases of diarrheal illness occurring worldwide annually, non-typhoidal *Salmonella* is estimated to be responsible for 3%, or 93.8 million of these cases (Majowicz *et al.*, 2010). Further, of the 93.8 million cases of *Salmonella* induced gastroenteritis occurring worldwide annually, approximately 155,000 result in death (Majowicz *et al.*, 2010).

Salmonella is an enteric pathogen that is usually acquired through the consumption of a contaminated food or water source. Once ingested, *Salmonella* makes its way to the intestines, where it can enter the epithelium via two routes. *Salmonella* enters the epithelium either via M cells, which are specialized cells in the intestinal epithelium that allow immune cells to sample antigens in the lumen, or through intestinal epithelial cells (Broz *et al*., 2012). After *Salmonella* has crossed the intestinal barrier, resident immune cells located in the epithelium, such as macrophages, will phagocytose the pathogen. Once inside these macrophages, *Salmonella* will either be degraded by lysosomal enzymes, or will use special virulence factors to survive and replicate in the cytoplasm of the macrophages (Broz *et al*., 2012). Following invasion, *Salmonella* is thought to induce an inflammatory diarrhea caused by the influx of neutrophils and subsequent disruption of intestinal

barrier function by neutrophil activity. Although *Salmonella* does induce inflammation, it is likely that before the onset of inflammation, *Salmonella*, like most other diarrheal pathogens, causes watery-diarrhea by altering the expression and/or function of intestinal ion transporters present throughout the epithelium (Zhang *et al.,* 2003). Previous work from our lab has shown that following *Salmonella* infection in mice, diarrhea develops before the onset of inflammation and is independent of neutrophil recruitment; infection in these mice does however eventually lead to inflammation and the influx of neutrophils. Here, we will focus on this initial phase of infection, before the onset of inflammation, where diarrhea is likely caused by alterations in transporter function.

The Role of Ion Transporters in Diarrhea

Ion transporters present throughout the intestines are responsible for the uptake of specific ions and nutrients, and function in the absorption of water (Supplementary Figure 1). The dysregulation of these transporters causes diarrhea (Field, 2003). The mechanism through which *Salmonella* causes changes in ion transport is not yet known. Elucidation of the mechanism(s) through which *Salmonella* causes diarrhea would aid in the development of novel therapeutics to combat *Salmonella* induced diarrhea.

Diarrhea occurs either as a result of increased secretion of ions or decreased absorption of ions by intestinal transporters and ion channels (Field, 2003 and Barrett, 2016). An excess of ions present in the lumen of the intestines will cause an outflow of water from the intestines into the lumen. The

mechanism by which diarrhea is induced is known for some enteric pathogens. A commonly studied diarrheal pathogen, *Vibrio cholera*e, causes diarrhea by increasing the activity of CFTR (Cystic Fibrosis Transmembrane Conductance Regulator), a channel protein present in the intestines that is responsible for secreting chloride. Increased chloride secretion, caused by *Vibrio cholerae,* causes an outflow of water from the intestines resulting in diarrhea (Kimberg *et al*., 1971, Sharp and Hynie, 1971, and Broeck *et al*., 2007). A more complete knowledge of similar downstream mechanism(s) behind *Salmonella* induced diarrhea is however still lacking.

In Vivo Models Used to Study Salmonella Infection: A Novel Nramp1 Wild-Type Model

In order to further study *Salmonella* induced diarrhea an effective model needs to be established. Two models used in the study of *Salmonella* infection are bovine models and murine models. The bovine model, widely used in the past, accurately capitulated the pathophysiology of diarrhea seen in humans. Although this model proved extremely useful to understanding the progression of diarrhea in *Salmonella* infection, it was not as amenable to genetic manipulation for use in mechanistic studies of *Salmonella* infection (Santos *et al.,* 2001). Murine models were therefore established to further investigate *Salmonella* infection. The initially established murine models however did not accurately recapitulate the pathophysiology of diarrhea seen during *Salmonella* infection in humans (Hapfelmeier and Hardt, 2005). Regular in-bred laboratory mouse strains infected with *Salmonella* develop severe

disseminated infection, with bacterial invasion into the bloodstream leading to a quick death. This occurs because most inbred mouse strains have a mutant *Nramp1* gene (Canonne-Hergaux *et al*., 1999). Wild-type *Nramp1* prevents *Salmonella* growth within the phagolysosome, preventing aberrant growth that can lead to sepsis (Canonne-Hergaux *et al*., 1999). Recently however, our lab developed a model using *Nramp1* wild-type mice, and found that when infected with *Salmonella* following kanamycin pretreatment to allow for sufficient colonization of *Salmonella*, these mice develop diarrhea similar to that seen in humans (Woo *et al.,* 2008). When infected with *Salmonella,* these Nramp1 wild-type mice showed decreased expression of the transporters DRA and ENaC (epithelial sodium channel) along with a redistribution of CFTR to the cytosol. The expression of transporters NKCC1 (Na^+-K^+-$2Cl^-$ cotransporter) and NHE3 (Sodium-hydrogen exchanger 3) were unchanged (Marcheletta *el al*., 2013). Of these changes in ion transport, decreased expression of DRA provides a potential explanation for *Salmonella* induced diarrhea. DRA is a chloride bicarbonate exchanger responsible for absorbing chloride; therefore decreased DRA would cause decreased absorption of chloride. This would lead to excess chloride build-up in the lumen of the intestines, resulting in diarrhea. Changes in DRA expression following *Salmonella* infection, therefore warrant further investigation.

Although experiments using *Nramp1* wild-type mice to study *Salmonella* infection are effective, these experiments tend to be expensive and time

consuming. In addition, although mouse models are amenable to genetic manipulation, which is important in mechanistic studies, the manipulation of specific pathways in mice following infection is more complicated. Therefore, an alternate *ex-vivo* model that accurately mimics intestinal physiology and pathophysiology in the setting of diarrhea would be very useful to study *Salmonella* infection.

In Vitro Models Used to Study Salmonella Infection: A Novel Enteroid Model

Although in vitro models are used to study *Salmonella* infection, these models do not completely reflect intestinal physiology. A widely used cell line in the study of enteric infection is the Caco-2 cell line (heterogeneous human epithelial colorectal adenocarcinoma). When made into monolayers and allowed time to reach confluence, these cell lines do develop into a brush border, but only contain one cell type, enterocytes (Delie and Rubas, 1997). The intestinal epithelium is composed of four main cell types: Paneth cells, which secrete antimicrobial peptides, goblet cells, which secrete mucus, enteroendocrine cells, which secrete various hormones, and enterocytes, which mediate absorption of electrolytes and other solutes, such as nutrients (Cheng *et al.,* 1974)(Supplementary Figure 2). These four cell types reside in different areas along the crypt-villus axis of the intestines, and stem cells located at the bottom of the crypts give rise to these four different cell types based on signals received from the Wnt and NOTCH signaling pathways (Pin *et al*., 2012)(Supplementary Figure 3). Until recently, it has been challenging

for in vitro models to accurately mimic this intestinal morphology. However, a new model involving the maintenance of intestinal stem cells in “organoids” that generate the differentiated lineages of epithelial cells listed above provides a useful solution to this problem.

Organoids are unlike other *in vitro* models because they accurately model the crypt-villus axis of the intestines, and contain the four different cell types of the intestinal epithelium. These organoids are produced from intestinal stem cells plated in Matrigel (basement-membrane matrix) to create 3D clusters of stem cells and their progeny that mimic the crypt-villus axis of the intestines (Sato *et al*., 2011). Organoids, also called “enteroids,” are maintained through serial passages in a proliferative state via the use of inhibitors that prevent differentiation. Enteroids can be differentiated into the four different cell types and made into enteroid-derived monolayers (EDM) to allow for an apical and basolateral polarization, which allows for apical infection with *Salmonella* (Miyoshi and Stappenbeck, 2013 and Foulke-Abel *et al*., 2016). Therefore organoids derived from mice present an efficient and effective model to study *Salmonella* infection. Moreover, recently Dr. Das’ group and others have been successful in isolating and culturing enteroids from healthy human patients and patients suffering from inflammatory bowel diseases, which opens up the future possibility of studying the mechanisms behind *Salmonella* infection using human intestinal organoids. Enteroids are also amenable to both overexpression and down-regulation of gene

expression via lentiviral transduction, which will aid in future mechanistic studies.

Aims of The Research

In order to better understand the mechanism(s) behind *Salmonella* infection we looked at changes in the Wnt/NOTCH signaling pathway in addition to changes in DRA using the enteroid model. The Wnt and NOTCH signaling pathways are relevant to the study of *Salmonella* infection because these two pathways control cell fate decisions in the intestines, and it is possible that *Salmonella* alters these pathways to cause aberrant proliferation of secretory over absorptive cell types to cause diarrhea. Liu *et al.* recently showed that the *Salmonella* effector AvrA increases levels of Wnt signaling. In addition it is possible that regulation of the Wnt and NOTCH signaling pathway is related to regulation of DRA. D'Angelo *et al.* showed that that the transcription factor HNF1α was responsible for regulating both Wnt/NOTCH signaling and DRA expression in the intestines (Liu *et al.,* 2012 and D'Angelo *et al.,* 2010).

Although various transporters are involved in the control of fluid absorption in the intestines (Supplementary Figure 1), we will focus specifically on DRA. As previously mentioned, *Marcheletta et al*. showed that expression of DRA was decreased in mice following infection with *Salmonella*. DRA is of interest in *Salmonella* infection because it has previously been implicated in other diarrheal diseases. Mutations in the DRA gene are responsible for causing diarrhea in CCLD (Congenital-Chloride Losing Diarrhea), a genetic

disorder characterized by severe high chloride content diarrhea (Höglund *et al*., 1996) Similarly, DRA knockout mice exhibit high chloride content diarrhea, aberrant growth of the intestinal mucosa, and crypt cell hyperplasia (Schweinfest *et al*., 2006).

We hypothesize that EDM derived from both proximal colon and small intestine will show down-regulation of DRA in addition to alterations in Wnt and NOTCH signaling following infection with *Salmonella*. Previous studies have shown that *Salmonella* effectors are capable of stabilizing β-catenin of the Wnt signaling pathway (Liu *et al.,* 2012). It is therefore possible that *Salmonella* infection can bias differentiation of the intestinal epithelium toward a secretory cell type, by activation of Wnt and downregulation of NOTCH signaling. To elucidate DRA expression and any alterations in the Wnt and NOTCH signaling pathway, we used EDM as a model for *Salmonella* infection and measured downstream changes in Wnt and NOTCH signaling, and DRA expression via RT-PCR, western blotting, and confocal microscopy.

MATERIALS AND METHODS

Isolation of Enteroids From Murine Intestine

Female mice aged 5-6 weeks were rendered unconscious via CO_2 and euthanized by cervical dislocation. The tissues of interest were dissected from these mice either from the distal end of the small intestine (ileum), a few inches from the cecum, or the proximal end of the colon, a few inches past the cecum, and placed in DMEM/F12 with HEPES (Sigma, St. Louis, MO) with added 10% FBS (Life Technologies, Gaithersburg, MD), 1% penicillin/streptomycin (100µg/ml, Sigma), 1% Glutamax (2mM, Life Technologies) (wash buffer). Tissues were opened longitudinally and scraped with forceps to remove villi and then subsequently washed in ice cold PBS (Life Technologies). Fat and connective tissue were removed from the tissue segments, and tissues were then minced thoroughly. 1ml of collagenase I solution (2mg/ml, Invitrogen, Carlsbad, CA) was added to the minced tissues and pipetted up and down; the mixture was incubated at 37°C for 10 minutes. The collagenase digestion step was repeated until individual crypts could be visualized under a microscope (digestion time was 40 minutes maximum). 10ml of wash buffer was added to de-activate collagenase I, and the mixture was filtered using a 70-µm cell strainer (Corning, Salt Lake City, UT). The mixture was centrifuged at 100-200g and isolated crypts were suspended in laminin-rich Matrigel (Corning). The Matrigel mixture was plated on either 24-well or 12-well plates (USA Scientific, Ocala, FL), inverted and allowed to

incubate at 37°C for 10 minutes as described in Miyoshi *et al*., before addition of 50% conditioned media (CM) containing Wnt3a, R-spondin, and Noggin to promote growth, along with inhibitors of Y27632 and SB431542 (inhibitors of ROCK and TGF-β, respectively)(Selleck Chemicals, Houston, TX) to prevent differentiation as described in Sato *et al*. (Miyoshi *et al.,* 2013 and Sato *et al*., 2009).

Maintenance of Organoids/ Generation of Enteroid-Derived Monolayers

Organoids derived from ileum and proximal colon were maintained as described above; suspended in laminin rich Matrigel in 50% CM with inhibitors of Y27632 and SB431542. These organoids were trypsinized and maintained through serial passages. In order to trypsinize organoids, 50% CM was removed and cells were scraped in cold 0.5 mM PBS-EDTA and spun down at 200g. 0.025% Trypsin (Life Technologies) was added to the cell pellet based on pellet density, and incubated at 37°C for 3 min. An appropriate volume of wash buffer (twice the volume of Tryspin added) was used to deactivate Trypsin. The mixture was spun down at 200g, and pellet was resuspended in Matrigel. 50% CM with inhibitors of Y27632 and SB431542 was added to the suspension of cells after 10 minutes incubation of inverted Matrigel plate at 37°C. Before infection, these enteroids were converted into EDM by trypsinizing as described above, and isolating single cells in 5% CM lacking SB431542 inhibitor. Cells were counted, and approximately 2 x 10^5 cells were added to permeable 6.5mm transwells (Corning) after coating the transwells with diluted Matrigel (1:30 in PBS) for 30min- 1 h. For western blots, 5 x 10^5- 1

x 10^6 cells were added to coated 12mm transwells (Corning). EDM were allowed to differentiate for two days in 5% CM media lacking SB431542 inhibitor and Wnt3a before infection.

Bacteria

Salmonella enterica serovar typhimurium strain SL1344 was used for infection of EDM generated on transwells and RFP-tagged SL1344 was used for infection of EDM generated on 8-well chamber slides (Fischer Scientific, San Diego, CA) for confocal microscopy. For culture, bacteria were inoculated into Luria-Bertani (LB) broth and grown for 8 h under aerobic conditions in an orbital shaker at 150rpm with appropriate antibiotics as done previously (Das *et al.,* 2011). The OD of the culture was taken using a spectrophotometer, and the appropriate amount of bacterial culture was collected and suspended in 1ml of was buffer without penicillin/streptomycin in order to get 5 x 10^8 colony forming units (CFU).

Infection

Bacteria were added to EDM at a multiplicity of infection (moi) of 10:1. After 1 h of invasion, cultures were washed and treated with gentamicin (50µg/ml, Life Technologies) for 90 minutes to kill remaining extracellular bacteria. Gentamicin was removed, and media was changed to 5%CM before collection of cell lysates at 6 or 24h. TER (transepithelial resistance) was measured at various time-points throughout infection and recorded as $\Omega*cm^2$.

Real-time Polymerase Chain Reaction

Cell lysates were collected from infected EDM at the 6 h time point using Lysis Buffer (Quiagen, Valencia, CA) by following the manufacturer's instructions. The lysate was placed onto an RNeasy column (Quiagen) and RNA isolated following the manufacturer's instructions. RNA was made into cDNA using qScript cDNA supermix (Quantabio, Beverly, MA) following the manufacturer's instructions and stored at -20°C. cDNA was then used to run polymerase chain reaction using a high capacity kit (Applied Biosystems, Carlsbad, CA) following the manufacture's protocol. Primers used are listed in Supplementary Table 1. All reactions were performed using the Step-One Plus system (Applied Biosystems). Target genes were normalized to β-actin. The resulting Ct values were calculated using single delta Ct as $2^{(\beta\text{-actin-Target})}$ and expressed in units relative to control.

Western Blot

At a 6 h timepoint EDM were lysed in cold RIPA buffer (50 mM Tris-HCl, 150 mM sodium chloride, 1mM EDTA, 0.25% sodium-deoxycholate, 1% NP40) with protease inhibitor and phosphatase inhibitor (Sigma) for 15 minutes on ice. After lysis, the samples were centrifuged and the supernatant collected. Equivalent amounts of protein for each sample were run on 10% polyacrylamide gels and then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). After transfer, the blot was blocked in either 5% non-fat milk (Lab Scientific Inc., Livingston, NJ) or 5% bovine serum albumin (Sigma) in phosphate-buffered saline containing 0.05% Tween-20 for 1 h at room temperature and incubated with rabbit anti-Down-Regulated in Adenoma (DRA

1:1000, Cell Signaling Technologies, Danvers, MA), rabbit anti- Notch intracellular domain (NICD, 1:1000, Cell Signaling Technologies) or mouse anti- β-actin (1:2000, Sigma) at 4°C overnight. Appropriate secondary antibodies, anti-rabbit IgG (1:2000, Cell Signaling) or anti-mouse IgG (1:2000, BD Biosciences, San Jose, CA) were added for 1.5 h at room temperature the following day, and the blot processed according to standard protocols with either SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Fempto Chemiluminescent Substrate (Thermo Fischer, San Diego, CA) and subsequent exposure to audioradiography film in a dark room (Genessee Scientific, San Diego, CA) for times ranging from 1 second- 5 minutes before developing. Densitometry was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

Confocal Microscopy

EDM were generated on 8-well chamber slides. At a 6 h time point cells were fixed for 15 minutes in ice cold 4% paraformaldehyde pH 7.2-7.3 (Wako Chemicals, Richmond, VA) supplemented with 10% FBS. After fixation cells were permeabilized with 0.1% TritonX-100 in PBS and incubated with primary antibodies to DRA (1:80, Sigma) and ZO-1 (Zonula occludens-1, 1:50, Cell Signaling) overnight and secondary antibodies (Alexa Fluor 488 donkey anti-rabbit 1:800 and DRAQ5 1:500) for 1.5 h before mounting with ProlongGold (Life Technologies). Imaging was performed using a Leica confocal microscope (Leica DMI4000B).

Periodic-Acid Schiff Staining

PAS staining was used to stain for goblet cells. EDM were generated on 8-well chamber slides. At a 6h time point cells were fixed in 100% ethanol for 1 minute and subsequently stained using Periodic acid-Schiff (PAS) kit (Sigma) following the manufacturer's instructions.

RESULTS

The generation of enteroid-derived monolayers from organoids

In order to establish our enteroid model, proximal colon and ileum were harvested from mice and used to isolate intestinal crypts. Using cutting edge stem cell based techniques, Lgr5+ stem cells present in the crypts were maintained as spheroids (Figure 1A). These spheroids were maintained undifferentiated through serial passages, until they reached an optimal density, and enough cells were available for making monolayers. Once the spheroids reached an ideal density, they were dissociated into single cells and differentiated into monolayers (enteroid-derived monolayers, EDM) on permeable transepithelial supports (Figure 1B). In order to test if the monolayers were differentiated, RT-PCR was used to test for Lgr5+ expression in both spheroids and monolayers (Figure 1C). EDM had lower Lgr5+ levels compared to spheroids, confirming that differentiation had occurred. Differentiated EDM were then used for infection.

***Salmonella* infection of enteroid-derived monolayers disrupts tight junctions and epithelial barrier function**

We first looked at how the epithelial barrier function of our monolayers was altered following *Salmonella* infection. EDM were infected with *Salmonella* and tight junction function was assesed via confocal microscopy for the tight junction protein ZO-1. Confocal microscopy showed that ZO-1 (green) was localized to the cell boundaries in uninfected EDM, but upon infection with

Salmonella, ZO-1, although still localized to the tight-junction, also appeared irregular and partially internalized (Figure 2A and B). In addition to redistribution of ZO-1, we wanted to see if overall barrier function was impaired following infection. In order to do this, epithelial barrier function of the monolayers was assessed by measuring the transepithelial resistance (TER) at various timepoints following infection. Uninfected EDM maintained stable values for TER, whereas infected EDM had TER reduced by half by 6 h post infection (Figure 2C). These results indicate that *Salmonella* infection of EDM, results in an overall decrease in intestinal barrier function.

Salmonella infection of enteroid-derived monolayer results in decreased DRA expression

We then looked at whether or not *Salmonella* infection of EDM would cause a decrease in the chloride/bicarbonate exchanger down-regulated in adenoma (DRA) as previously seen *in vivo* in the proximal colon of mice. DRA expression was assessed via RT-PCR for transcript expression and western blots for protein expression in samples from both uninfected and infected EDM from both proximal colon and small intestine (Figure 3). DRA was found to be downregulated in infected EDM from both proximal colon and small intestine in both RT-PCR and western blot analyses. In addition to looking at the expression of DRA, we wanted to visualize the localization of DRA in EDM. DRA is an ion transporter, and in vivo it is present in the apical membranes of the cells of the villus, therefore, we expect to see DRA similarly localized around the cell boundary in EDM. Confocal microscopy using antibodies

against DRA showed that DRA (green) is localized around the outer border of cells in EDM generated from both colon and small intestine (Figure 4A). We also looked at the association of *Salmonella* with EDM (Figure 4B). Despite the ability to visualize DRA localization using confocal microscopy, we were unable to notice differences in the expression of DRA in uninfected versus infected EDM using this method of analysis. The inability to notice differences in expression may be due to a lack of polarization of EDM in 8-well chamber slides. Confocoal microscopy for DRA expression on polarized EDM generated on transwell inserts might provide more insight into DRA expression in EDM following *Salmonella* infection.

Salmonella infection of enteroid-derived monolayers increases goblet cell proportion

Salmonella effectors have been shown to stabilize the Wnt signaling pathway, one of the major pathways involved in determining cell fate commitment in the intestines (Liu *et al.,* 2012). In addition, studies have also linked the regulation of the Wnt and NOTCH signaling pathway to the regulation of DRA expression (D'Angelo *et al.,* 2010). We therefore wanted to investigate whether infection with *Salmonella* alteres cell fate commitment in the intestinal epithelium. Previous testing of the secretory-cell type markers, Muc2 and ATOH1 in EDM derived from small intestine showed increases in expression in these markers on a transcriptional level in infected versus uninfected EDM derived from small intestine. Muc2 is a marker for goblet cells while ATOH1 is a general secretory cell type marker. Therefore, we performed

Periodic-Acid Schiff (PAS) staining to try and visualize any changes in goblet cell number and mucus production in our EDM following *Salmonella* infection (Figure 5). Preliminary data from PAS staining of EDM derived from small intestine show an increase in the proportion of goblet cells in infected EDM when compared to uninfected EDM.

Salmonella infection of enteroid-derived monolayers decreases NCID of the NOTCH signaling pathway

The increased proportion of goblet cells following infection of EDM with *Salmonella* suggested that there may be alterations in the Wnt and NOTCH signaling pathway. The previously observed increases in ATOH1 and Muc2 taken with the increased proportion of goblet cells following infection points to a potential bias toward secretory cell fate commitment following *Salmonella* infection. To further investigate any changes in Wnt and NOTCH signaling, we looked at NICD (Notch-intracellular domain) expression following infection of EDM with *Salmonella*. NICD is a downstream signaling molecule involved in NOTCH signaling. We found that NICD was decreased following infection of both colonic and small intestinal EDM (Figure 6), offering a potential explaination for both the decreases in DRA and increases in goblet cells seen.

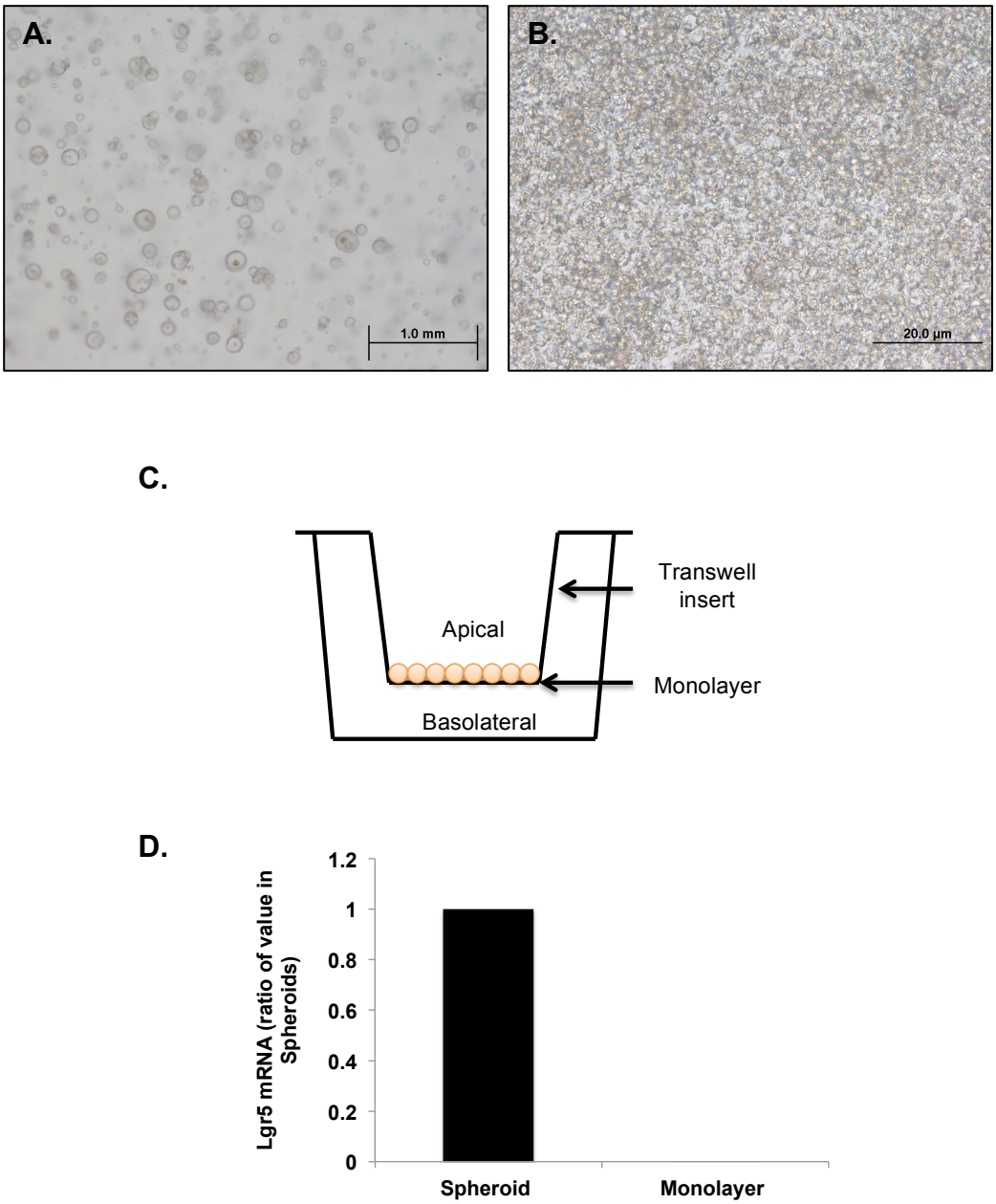


Figure 1: Differentiation of enteroids into EDM. Enteroids at day 2 after isolation from murine proximal colon (A). EDM derived from enteroids from murine proximal colon (B). Representative image of EDM generated on transwell inserts, infection with *Salmonella* occurs at the apical side of the monolayer (C). Lgr5, a stem cell marker, is decreased upon differentiation of enteroids into EDM (D). Data are from 2 independent experiments with a total n=2 for spheroid and n=6 for monolayer ($P<0.05$, Student's T-Test).

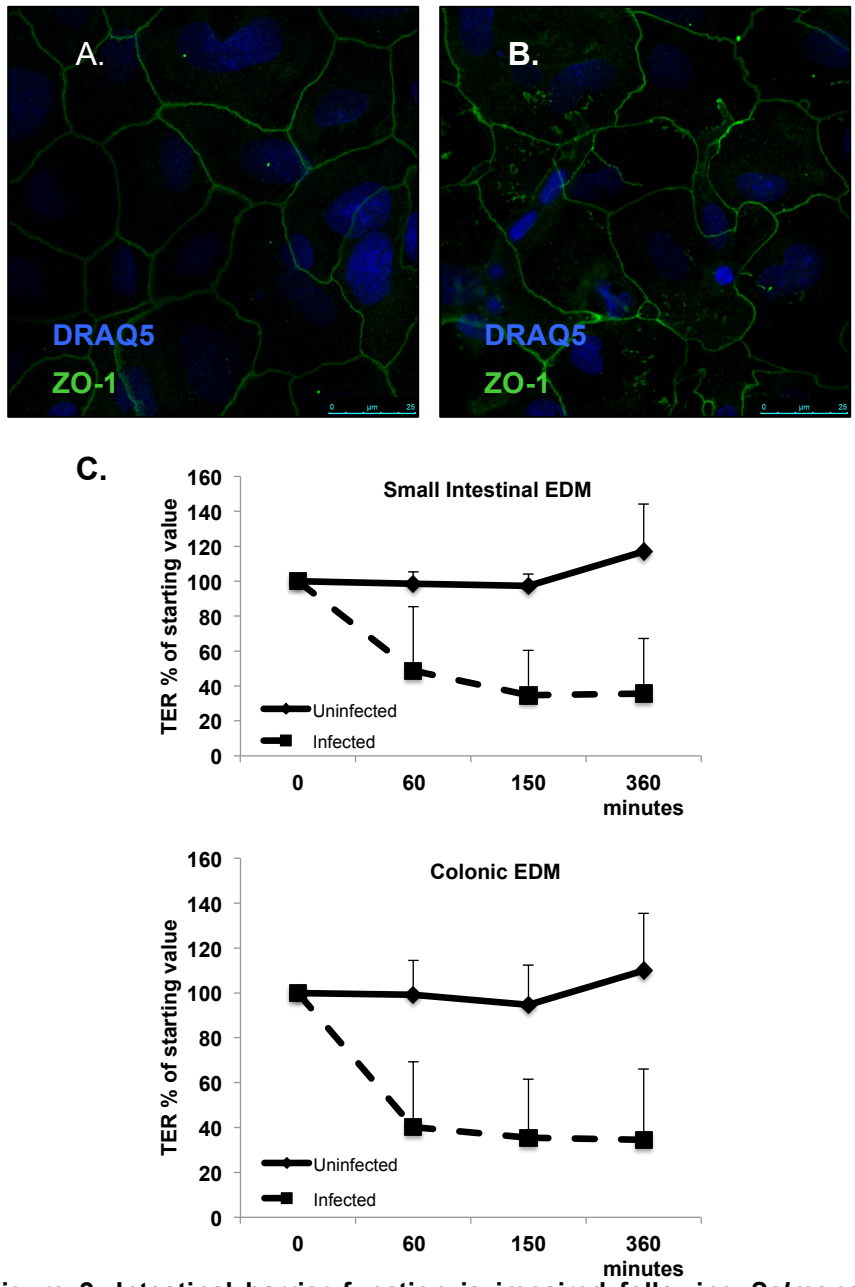


Figure 2: Intestinal barrier function is impaired following *Salmonella* infection. Small intestinal EDM were infected with *Salmonella* strain SL1344, and treated with gentamicin after one hour to kill remaining extracellular bacteria, maintained 6h samples were fixed and stained for confocal microscopy for the tight junction protein ZO-1 (green). Monolayers show disruption of tight junctions in *Salmonella* infected (B) verses uninfected samples (A). Data is from 1 experiment with n=4 for both uninfected and infected EDM. Transepithelial resistance (TER) measurements show a 50% reduction in barrier function in infected samples from both small intestinal and colonic EDM following 6h of infection (C). Data are from 4 independent experiments with a total n=8 infected for small intestinal EDM ($P<0.05$, Student's T-Test) and data are from 4 independent experiments with a total n=12 infected for colonic EDM ($P<0.05$, Student's T-Test).

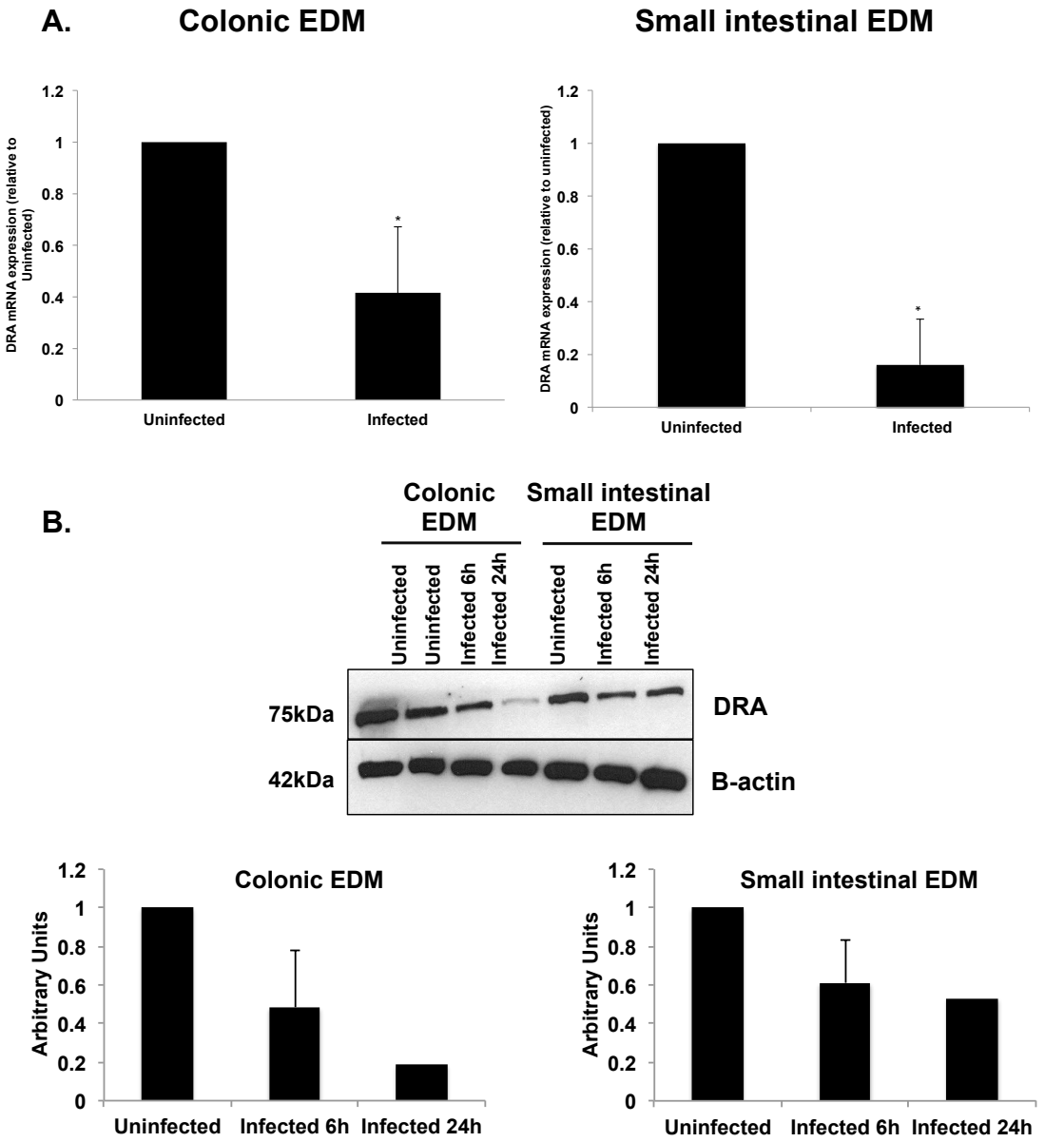


Figure 3: DRA is down regulated in both colonic and small intestinal EDM following *Salmonella* infection. EDM derived from mouse colon or small intestine were infected with *Salmonella* strain SL1344 and treated with gentamicin after one hour to kill remaining extracellular bacteria. Samples were lysed and collected at a 6 h time-point. Lysates were subsequently analyzed for DRA mRNA levels via RT-PCR (A). All uninfected samples were normalized to a value of 1. Data are from 5 independent experiments with a total n=9 infected for proximal colon. Data are from 7 independent experiments. n=12 infected for ileum (*P<0.05, Student's T-test). The cell lysates from uninfected and infected, colonic and small intestinal EDM, after 6 h and 24 h of infection were loaded on SDS-PAGE and immunoblotted with DRA antibody (B). Equal loading was checked by probing the blot for the loading control β-actin. The blot is representative of three independent experiments for 6 h, only one experiment contained a 24 h time point; densitometry combining experiments is shown below the blot. Data is significant for the 6 h time point in both colonic and small intestinal EDM (P<0.05, Student's T-Test).

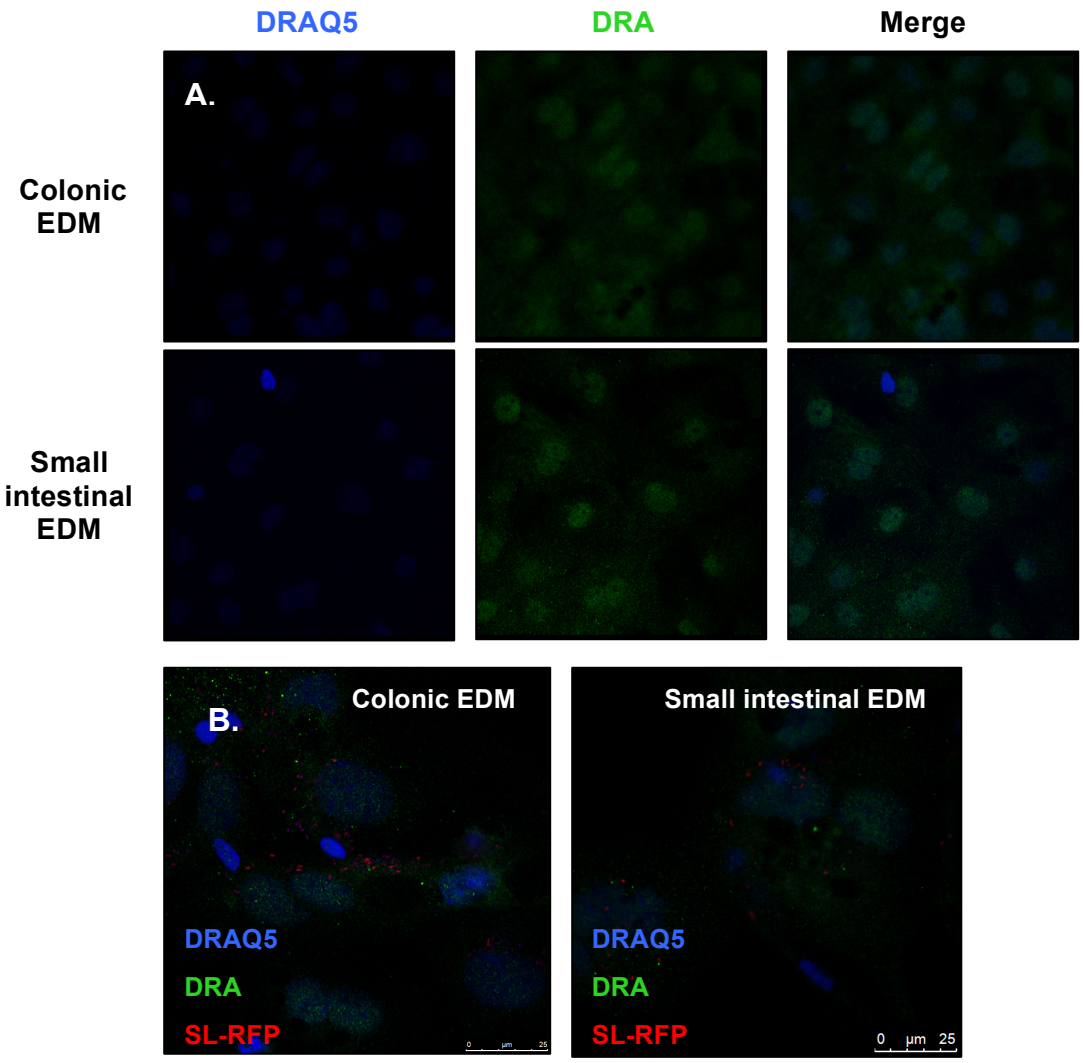


Figure 4: Immunofluorescence staining for DRA shows localization of DRA in EDM. Uninfected EDM from both colon and small intestine were fixed and stained after 6 h for DRA (green)(A). DRA is localized around the boundary of the cells. EDM were infected with *Salmonella* strain SL1344 tagged with RFP, and treated with gentamicin after one hour to kill remaining extracellular bacteria, maintained 6 h samples were fixed and stained for confocal microscopy for DRA (green)(B). *Salmonella* are associating with EDM at the boundaries of the cells, some *Salmonella* appear to be internalized.

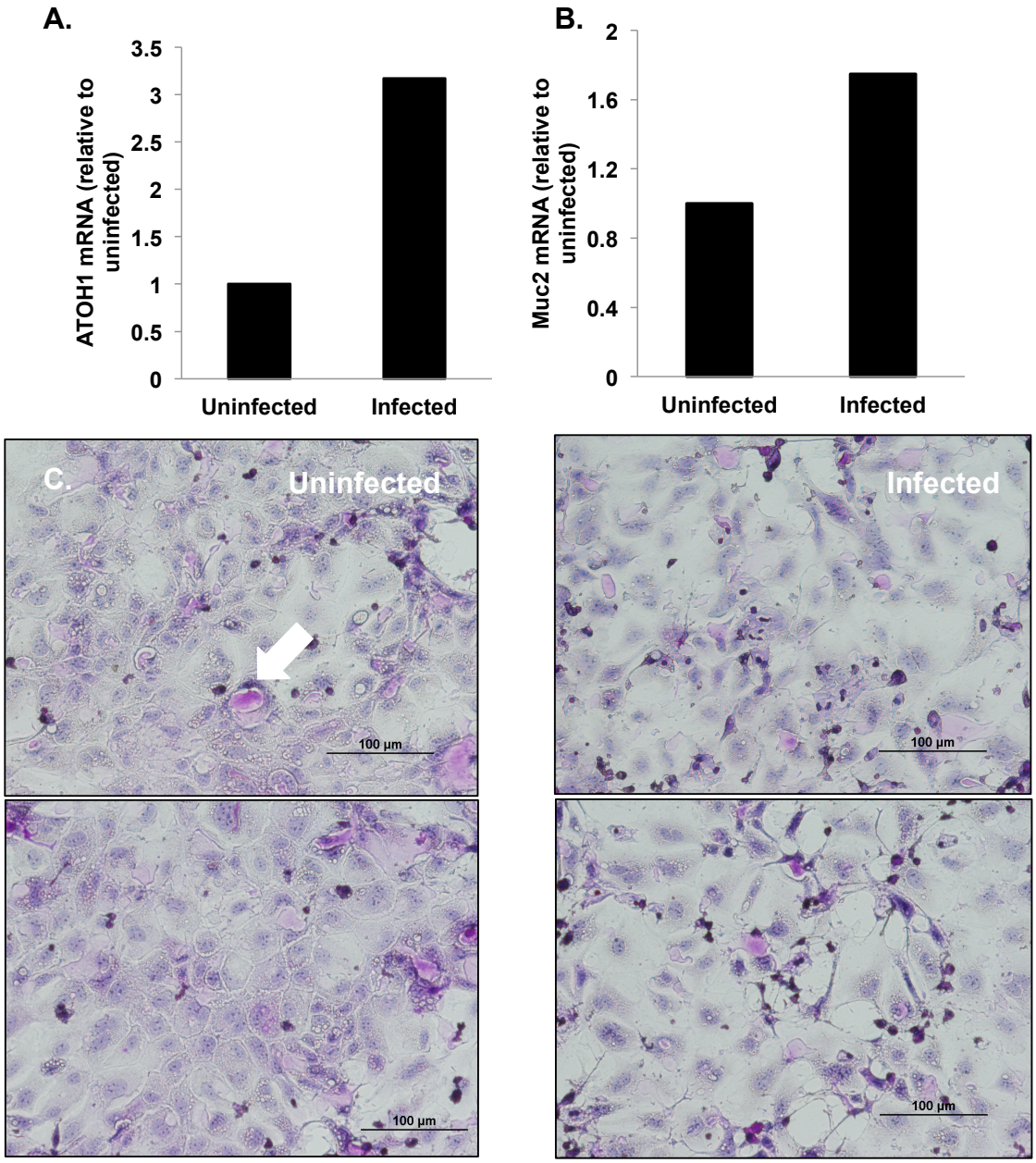


Figure 5: *Salmonella* biases differentiation of the epithelium toward secretory lineages. EDM, derived from mouse small intestine were infected with *Salmonella* strain SL1344 treated and treated with gentamicin after one hour to kill remaining extracellular bacteria. Samples were lysed and collected at a 6 h time-point. Lysates were subsequently analyzed for ATOH1 (A) and Muc2 (B) mRNA levels via RT-PCR. All uninfected samples were normalized to a value of 1. Data is from 2 independent experiments. EDM derived from mouse small intestine were infected with *Salmonella* strain SL1344 and treated with gentamicin after one hour to kill remaining extracellular bacteria. Samples were fixed and stained at 6 h using Sigma kit 395B-1KT for goblet cells. A representative goblet cell is indicated with a white arrow in the first panel of (C). Data is representative of 2 independent experiments.

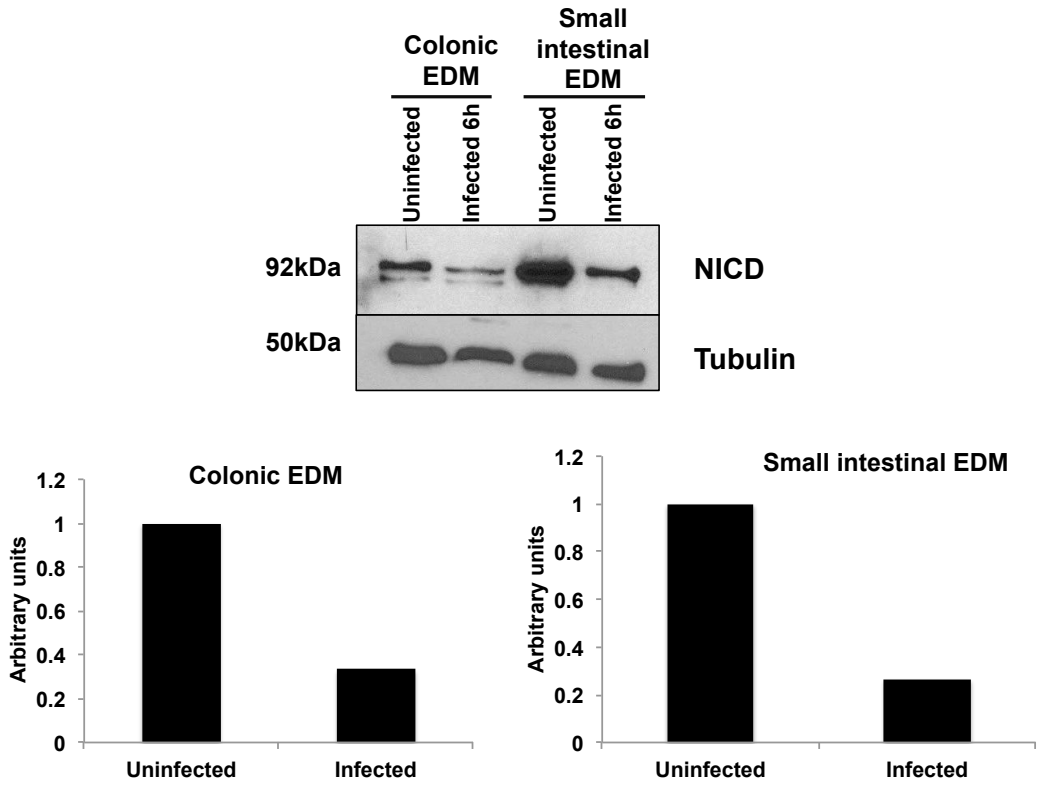


Figure 6: NICD is decreased following infection with *Salmonella* in both colonic and small intestinal EDM. EDM, derived from mouse proximal colon and ileum were infected with *Salmonella* strain SL1344, treated with gentamicin after one hour to kill remaining extracellular bacteria. The cell lysates from uninfected and infected, colonic and small intestinal EDM, after 6 h of infection were loaded on SDS-PAGE and immunoblotted with NCID antibody. Equal loading was checked by probing the blot for the loading control Tubulin. Densitometry is shown below the blot. Data is from 1 independent experiment.

DISCUSSION

Previous studies from our lab have shown that DRA is down regulated in the proximal colon of mice prior to increased levels of inflammation (Marcheletta *et al*., 2013). Here, we focus on DRA expression in EDM derived from both the proximal colon and small intestine of mice. We hypothesized that EDM generated from both colon and small intestinal would show similar decreases in DRA as seen *in* vivo accompanied by alterations in the Wnt/NOTCH signaling pathways. In this study, we have found that DRA was down regulated in EDM derived from both proximal colon and ileum following infection with *Salmonella* similar to the results seen in vivo in the proximal colon of mice. Decreased expression of DRA following *Salmonella* infection offers a potential mechanism for *Salmonella*-induced diarrhea. Excess chloride ions in the lumen of the intestines as a result of decreased DRA expression contribute to water loss in the intestinal epithelium. In addition to quantifying DRA expression, we also used confocal microscopy to visualize DRA localization in EDM. *In vivo* DRA is localized at the apical side of the epithelium in cells of the villus. Our data show that similarly, in EDM, DRA is localized around the border of the cells.

Confocal microscopy was also used to assess tight junction function following infection. In infected EDM, the tight junction protein ZO-1 appeared irregularly distributed at the tight junctional boundary between cells. ZO-1 was also slightly internalized in infected versus uninfected samples. Overall barrier

function assessed by TER measurements taken at time-points post-infection showed that infected EDM had significantly reduced barrier function by 1 h, consistent with the decreased ZO-1 seen via confocal microscopy. Decreased barrier function following infection of EDM could be attributed to the invasion of *Salmonella* into the epithelium. *In vivo*, *Salmonella* enter the intestinal epithelium either via M cells or invasion of epithelial cells through the use of T3SS-1 (type-three secretion system) effectors (Broz *et al*., 2012). A drawback to EDM as a model is that they lack M cells, in addition to immune cells usually present in the epithelium. Therefore in the EDM model, *Salmonella* most likely enter the cells solely via the use of T3SS-1 to manipulate cellular actin. This method of entry would account for altered ZO-1 localization, and impaired barrier function. Decreased barrier function leads to leaky tight junctions, which contribute to luminal fluid accumulation. During *Salmonella* infection *in vivo* inflammation occurs at a later stage after the onset of diarrhea, increased levels cytokines during this stage causes the recruitment of neutrophils, which in their efforts to contain infection cause damage to the tight junctional boundary (Broz *et al*., 2012). Because EDM lacks neutrophils, tight junctional deregulation most likely occurs as a result of invasion of *Salmonella.*

In addition to changes in DRA expression, we looked at alterations in the Wnt and NOTCH signaling pathways following infection. The Wnt and NOTCH signaling pathways are responsible for controlling cell-fate commitment in the intestines, and it is likely that infection with *Salmonella*

alters these pathways. The alteration in these pathways may be linked with the down- regulation seen in DRA. Previous analysis of the secretory cell type marker ATOH1 and goblet cell specific marker Muc2 showed increased expression following *Salmonella* infection of small intestinal EDM. PAS staining of EDM similarly shows what appears to be an increased proportion of goblet cells. We next looked at whether or not increased goblet cell proportion was accompanied by changes in the Wnt and NOTCH signaling pathways. Expression of NICD, the downstream NOTCH signaling molecule, was decreased following infection of both colonic and small intestinal EDM. This is consistent with the observation that inhibition of NOTCH signaling leads to goblet cell hyperplasia (van Es *et al.,* 2005). The interplay between the Wnt and NOTCH signaling pathway in the intestines is however complex. Increases in NOTCH signaling have been shown to result in no changes in certain components in the Wnt signaling pathway (van Es *et al.,* 2005). In addition, AvrA, a *Salmonella* effector has been shown to cause up regulation of the Wnt signaling pathway, and other studies have shown that β-catenin can bind and activate Jagged1, a NOTCH ligand, leading to the activation of NOTCH signaling (Liu *et al.,* 2012 and Rodilla *et al.,* 2009). Preliminary data investigating the expression of both β-catenin and phospho- β-catenin following *Salmonella* infection in EDM show no significant changes to this pathway in uninfected versus infected EDM. In regards to Wnt and NOTCH activation in our EDM model, further investigation into the expression of the

Wnt signaling pathway will help to elucidate the interplay between these two pathways during *Salmonella* infection.

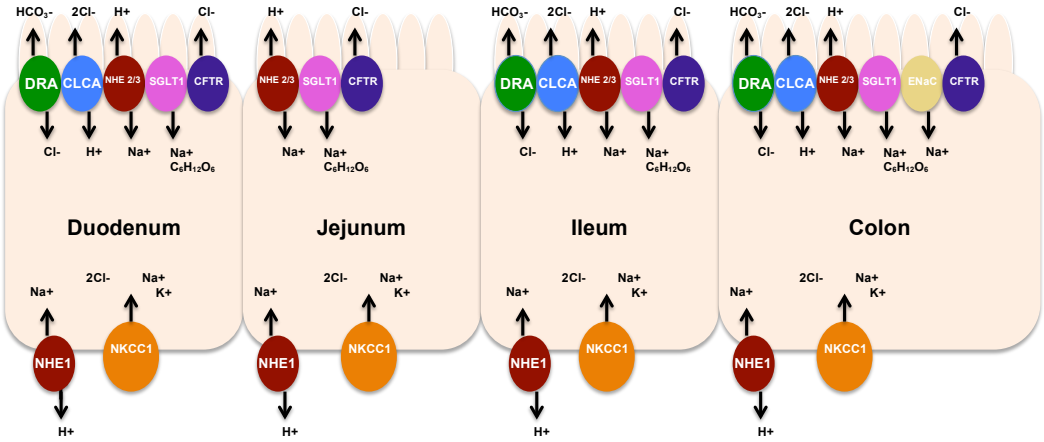
The decreased NCID in EDM following infection could account for DRA down-regulation. Loss of NCID results in an inability of the intestinal epithelium to differentiate into absorptive cell types, and this could indirectly cause decreased transporter expression. It is also likely that loss of absorptive cell types is accompanied by decreases in DRA directly caused by *Salmonella* effectors. These two events could occur simultaneously, therefore further investigation is needed to determine whether decreased absorptive lineages is the only factor accounting for DRA down-regulation. In order to do this, γ-secretase inhibitors could be used to block NOTCH signaling, and subsequently any downstream changes in DRA expression can be measured. The extent of DRA down-regulation as a result of γ-secretase inhibitors can be compared to the extent of DRA down regulation seen in *Salmonella* infection to determine if γ-secretase inhibition is the only mechanism contributing to DRA down regulation following *Salmonella* infection. If specific *Salmonella* effectors are contributing to DRA down-regulation, effector mutants could be used to test the role of individual effectors in causing DRA down-regulation.

Another potential connection between the decreases seen in DRA, and increases in goblet cell number accompanied by decreased NOTCH signaling are the transcription factors Hepatocyte nuclear factor 1α and β. D'Angelo et al showed that mice deficient in HNF1α and β display increases in goblet cells along with decreases in DRA. Mice deficient in HNF1α and β also showed

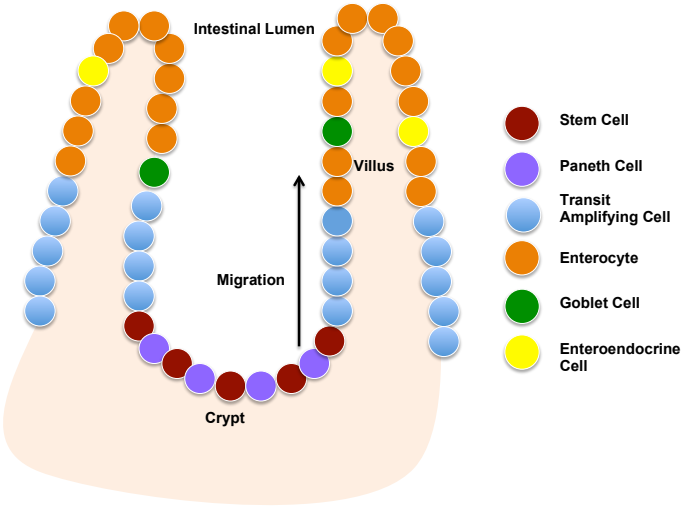
decreases in both the NOTCH and Wnt signaling pathways (D'Angelo et al., 2010). Preliminary data investigating the expression of HNF1α following infection of both colonic and small intestinal EDM however show no differences between uninfected and infected conditions. Although further investigation is needed, this suggests that there may be another mechanism at play that connects the decreases in DRA with increased goblet cell number and decreased NICD.

In regards to potential therapeutics for *Salmonella* induced diarrhea, if DRA is the major cause of *Salmonella* induced diarrhea, then up-regulation of DRA should abrogate diarrhea. In future experiments, we plan to overexpress DRA in mice to determine whether or not this overexpression can reverse *Salmonella* induced diarrhea. If in fact overexpression of DRA can treat *Salmonella* induced diarrhea, compounds such as all-trans retinoic acid, which have been shown to increase DRA expression, could be used as a new form of therapeutic (Priyamvada *et al.,* 2015).

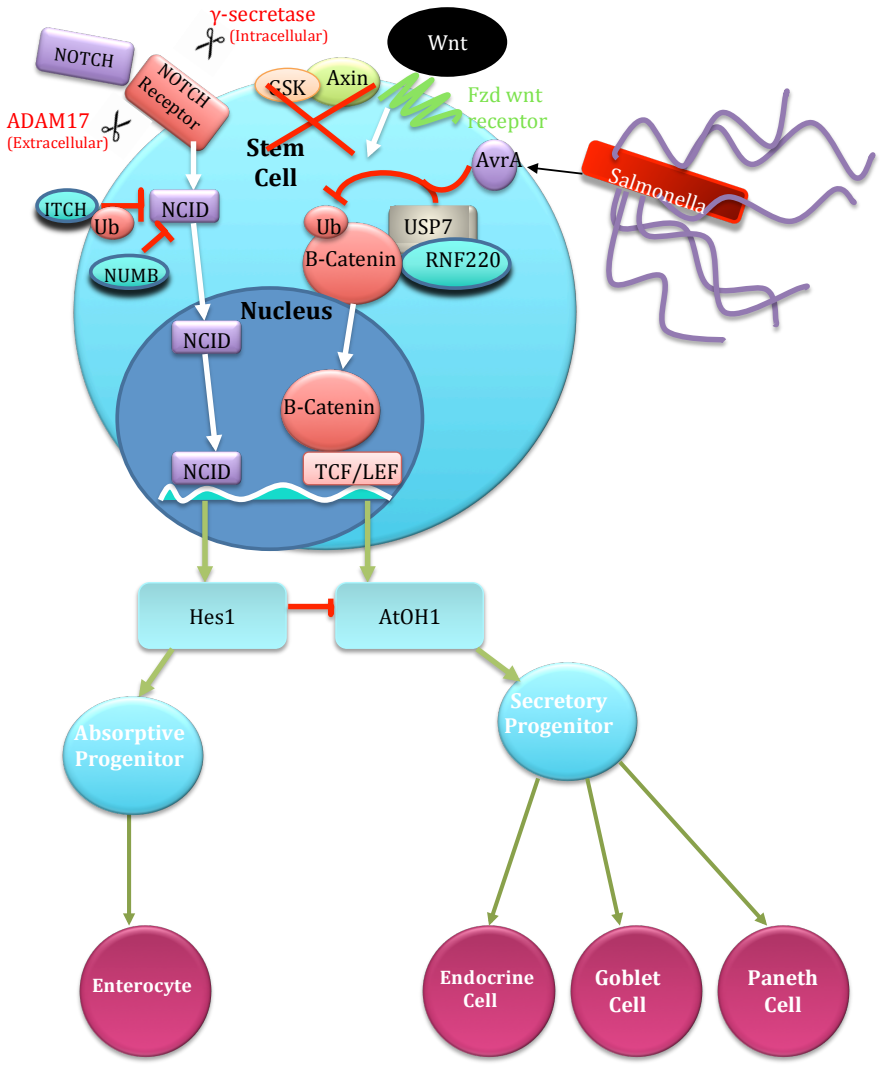
In conclusion, following *Salmonella* infection EDM show decreased intestinal barrier function, accompanied by decreases in the chloride/bicarbonate exchanger DRA, increased goblet cells, and decreased NOTCH signaling. Although the model is limited by the lack of immune cells, EDM provide a useful tool for further studying the mechanisms of *Salmonella* infection on the intestinal epithelium.



Supplementary Figure 1: Ion transporters present throughout the intestines aid in the absorption of ions and nutrients. The three sections of the small intestine are shown: duodenum, jejunum, and ileum. The large intestine is shown as the colon. Ion transporters are present at both the apical (DRA, CLCA, NHE2/3, SGLT1, and CFTR) and basolateral surfaces (NHE1 and NKCC1). The ions absorbed and/or secreted by each transporters are depicted via arrows either leading out of or into the intestinal epithelium.



Supplementary Figure 2: The organization of the crypt-villus axis. Lgr5+ stem cells present in at the base of the crypts give rise to the four main cell-types present in the intestines. Cell fate commitment is based upon signals received from the Wnt and NOTCH signaling pathways.



Supplementary Figure 3: The Wnt and NOTCH signaling pathways control cell fate commitment in the intestines. In the NOTCH pathway binding of NOTCH ligands cause extracellular and intracellular cleavage of the NOTCH receptor. The intracellular fragment (NCID, Notch Intracellular Domain) translocates into the nucleus, where it causes the expression of a transcription factor, Hes1, which subsequently inhibits AtOH1 of the Wnt signaling pathway. Activation of NOTCH signaling gives rise to absorptive progenitors. ITCH and NUMB are negative regulators of the NCID causing its ubiquitin-mediated degradation. In the Wnt pathway, activation of the Wnt receptor stabilizes β-catenin, preventing its ubiquitination and subsequent degradation by the GSK/Axin complex. β-catenin is then translocated into the nucleus where is deactivates transcriptional repressors TCF and LEF, allowing the genes for ATOH1 to be transcribed. Activation of ATOH1 leads to downstream differentiation of the stem cells into secretory cell types.

Supplementary Table 1: Sequence of Primers Used for Expression Analysis (RT-PCR)

DRA Forward:	AGG GAA TGC TGA TGC AGT TTG CTG
DRA Reverse:	AGT TGA AAT GCT ACA CTT GCC GCC
ATOH1 Forward:	CCG TCA AAG TAC GGG AAC AG
ATOH1 Reverse:	GAG TAA CCC CCA GAG GAA GC
Muc2 Forward:	GAA GCC AGA TCC CGA AAC CA
Muc2 Reverse:	GAA TCG GTA GAC ATC GCC GT
β-actin Forward:	GAC GGC CAG GTC ATC ACT AT
β-actin Reverse:	ACA TCT GCT GGA AGG TGG AC

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