Role Of The Immune System In The Modulation Of The Mmr-Deficient Intestinal Stem Cell Niche

Shepard Conner

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ROLE OF THE IMMUNE SYSTEM IN THE MODULATION OF THE MMR-DEFICIENT
INTESTINAL STEM CELL NICHE

by

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ROLE OF THE IMMUNE SYSTEM IN THE MODULATION OF THE MMR-DEFICIENT INTESTINAL STEM CELL NICHE

A Thesis

Presented to the Faculty of

The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science

by

Shepard Colby Conner, B.A.
Houston, Texas

December 2023
Mismatch Repair (MMR) is a crucial DNA repair system to maintain genomic integrity in cells that is integrated by specific genes including MLH1, MSH2, MSH6, and PMS2. These genes play a critical role in repairing errors that occur in base pairing by stabilizing the genetic material. When the MMR system fails to correct those errors, MMR deficiency occurs where monoallelic mutations in the MMR genes result in a condition known as Lynch Syndrome (LS). LS makes up approximately 3% of all colorectal cancer (CRC) and is regarded as a hereditary form of CRC, which progresses from MMR-deficient (MMRd) intestinal stem cells (ISCs). Many studies have shown that the immune system plays a critical role in influencing the genetic expression of stem cells. Our lab has also shown that naproxen, a non-steroidal anti-inflammatory drug (NSAID), has been effective at activating different subtypes of immune cells including macrophages. The purpose of this study was to understand how the immune cell compartment interacts with MMRd cells within the ISC niche. In this study, biopsied normal colorectal mucosa from MMRd patients after 6 months of exposure to naproxen was stained with stem cell and differentiation biomarkers using an in situ hybridization assay to quantify the marker density. Colon samples from MMRd mice were also collected and grown as organoids. The interactions between ISCs and the immune system were observed via changes in gene expression by culturing organoids with macrophage secretome. Mass Spectrometry was also used to identify factors in the macrophage secretome that may have contributed to the observed changes. Overall, results indicate that there is a significant increase in the quantification of stem cell biomarkers in MMRd normal mucosa after daily exposure to naproxen. There are also secreted factors present in the macrophage secretome, which are involved in the regulation of cellular proliferation and apoptosis thus validating our findings that the immune system modulates the MMRd ISC niche.
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Introduction

Colorectal Cancer (CRC) is the third most prevalent cancer in the world, afflicting about 1.8 million people worldwide. Not coincidentally, CRC is also the third leading cause of death attributed to cancer in the United States (Tanakaya et al. 2020). Lynch Syndrome (LS) is the most common form of hereditary colorectal cancer, making up 3% of all CRCs with an estimated near 1 million carriers in the United States (Kastrinos et al., 2017). LS is a hereditary cancer that increases the risk of colorectal cancer and predisposes to the development of tumors of the endometrium, ovary, and urothelial tract, among others. LS follows existing germline mutations in one of four DNA mismatch repair (MMR) genes (MLH1, MSH2, MSH6, and PMS2). This secondary mutation causes a deficiency in repairing insertion-deletion mutations (indels) (Watson et al., 2008). These DNA mismatch repair genes are essential for identifying and repairing errors in DNA replication such as insertions, deletions, and incorrect base pairing between strands of DNA, as well as repairing DNA damage overall to stabilize the genetic material (Watson et al., 2008).

These MMR genes determine the status of a cell’s capability to undergo mismatch repair, either being MMR proficient (MMRp) or MMR deficient (MMRd). In CRC, 15-18% of cancers as a whole are MMRd expressed through microsatellite instability (MSI-H). While the majority of these CRCs acquire this deficiency through somatic mutation events such as hypermethylation of the MLH1 promoter, around 3% of CRCs acquire MSI-H through a germline mutation in one of the four MMR genes in addition to a somatic alteration in the alternative allele of the same MMR gene (Vilar and Gruber, 2010). This
leads to the subsequent accumulation of widespread genomic MSI-H where microsatellite alleles gain and lose repeat units at a frequency higher than the rate of normal cells (Yu et al., 2013). Microsatellite instability is therefore a known indicator of impaired DNA MMR function and by extension is an indicator of LS where microsatellites, a genomic structure that contains repeated nucleotides, are unstable because they are prone to form hairpins and other complex structures (Schlotterer and Harr, 2004). These nucleotides are typically observed as repeats of mono-, di-, tri-, or tetranucleotide units with repeats up to 100 times (Schlotterer and Harr, 2001). During the synthesis phase of DNA replication, an error in the process creates an MSI region in long repetitive sequences of DNA due to DNA polymerase slippage where the DNA polymerase III holoenzyme becomes displaced from the template strand (Canceill et al., 1999). This slippage is responsible for creating an insertion-deletion loop that is normally able to be recognized and subsequently repaired by the MMR system. If the MMR system does not function properly, during the second round of replication the daughter strand is synthesized incorrectly, resulting in the daughter strand containing frame-shift mutations. As a result of the frame-shift mutation, a nonsense mutation forms downstream of the error which results in the translation of a protein that cannot carry out its intended function (Boland and Goel, 2011).

The issue with MMRd in colorectal cancer is that MMRd intestinal stem cells (ISCs) in the crypts of the colon progressively differentiate into epithelial cells where they have the ability to ignore cell cycle signaling (Stoian et al., 2016). Ignoring cell cycle signaling can lead to uncontrolled cell growth as the time at which cells enter different such as synthesis or growth is not regulated. LS presents a model to study carcinogenesis in a
DNA MMRd setting where a common underlying feature is MMRd ISCs that contain downregulated ISC-specific genes. (Bommi et al., 2021). These ISC genes are known to be expressed by stem cells in the normal colonic crypt structure (Baker et al., 2015). The differentiated cells that arise from these ISCs are known to ignore apoptotic signals and proliferate without control. As such, carcinogenesis results and leads to cancer progression in affected individuals.

Widespread genomic MSI, including instability in ISCs, is a known indicator of MMRd in cells (Yu et al., 2017). These ISCs are cells that have been characterized by their capability to be immortal due to their slow turnover rate and their ability to self-renew when functioning normally. These ISCs generate the normal colon mucosa which has a high rate of turnover and cellular proliferation, making cancer cells more prone to develop in this area through accumulating a higher mutation burden over time (Stoian et al., 2016). The ISCs are located at the base of the intestinal crypt in what is referred to as the stem cell niche and progressively differentiate, moving upwards into the crypt structure (Stoian et al., 2016). As a result, all cells within the intestinal crypt are believed to arise from a singular stem cell according to the Unitarian Theory (McDonald et al., 2006). Extending from this, if MMRd cells give rise to all other cells within the ISC niche, then those differentiated cells also have the capability to become MMRd due to the germline mutation in LS (Stoian et al., 2016). In other words, if a stem cell is cancerous, every cell differentiating from that crypt is also and will lack mismatch repair functionality, progressing to LS. In addition, cancer cells are capable of recruiting other cells through chemical and inflammatory stimuli to create a microenvironment that supports their growth and migration. Cancer associated fibroblasts, for example, have been shown to
secrete a number of proinflammatory cytokines and growth factors including CXCL12 and VEGF which allow the modified extracellular matrix (ECM) to support their stemness and survival (Pein and Oskarsson, 2015).

Stem cells and their multipotent potential have been shown to be influenced by non-steroidal anti-inflammatory drugs (NSAIDs) (Alaseem et al., 2015). Different types of stem cells such as type-10 collagen and mesenchymal stem cells in adipocytic differentiation have illustrated the effects NSAIDs have on gene expression alteration (Fredricksson et al., 2013). The Vilar research group showed that the NSAID, naproxen, was effective in decreasing inflammatory signals, a hallmark of cancer, by reducing prostaglandin levels via COX-1/COX-2 inhibition in the colorectal mucosa of LS patients (Reyes-Uribe et al., 2021). These NSAIDs are also relevant because inflammation is believed to cause microsatellite alterations within DNA which can alter cellular phenotype (Koi et al., 2018). Pathway enrichment analysis has been conducted and has determined that naproxen affects the enrichment signals corresponding within immune pathways. Furthermore, in silico analysis of deconvolution of mRNA-seq data has shown that naproxen has activated different subtypes of immune cells. These include T cells, B cells, and macrophages where positive enrichment of these cell types was found after naproxen exposure (Reyes-Uribe et al., 2021). The activation of these cell types is important as it shows that these types of immune cells were influenced by the NSAID exposure and therefore could be relevant when looking at the immune system’s role on the ISC.

The purpose of this study is to understand how the immune cell compartment interacts with MMRd cells within the ISC niche. In order to accomplish this, with the
knowledge from prior research about the effect naproxen has on activating immune cells, I seek to understand the interaction between immune cells and the ISC niche in a MMRd context. I believe that the immune cell compartment within the intestinal stem cell niche is involved in crosstalk signaling communication with ISCs in regulating their state thereby affecting their ability to renew and continue to differentiate. In order to design interventions to reduce the prevalence of LS, there needs to be an understanding of how MMRd is regulated in the stem cell niche environment. Knowing that naproxen interacts with immune cells, I seek to understand the effect activated immune cells have downstream on the MMR genes in modulating the ISC niche through their mechanisms including antigen presentation and clonal expansion.

**Literature Review**

The current literature in the field of MMRd is vast due to the prevalence of LS in the population of CRC patients. Recent studies including those that our lab has conducted have focused on and highlighted how MMRd influences the frequency of mutations and errors in sporadic CRC and LS CRC. One of the key findings has been that while instability of microsatellites (MSI-H) is an indicator of both LS and sporadic CRC, there is a higher frequency of MSI-H in LS colonic cancers. It has been acknowledged that greater than 90-95% of CRCs in LS carriers display MSI-H (Truta et al., 2008). This is compared to 15% of stage III sporadic CRCs (Petrelli et al., 2019). Determining MSI-H and MMRd has been proven to be assessed through two different methods to identify the target genes of MMRd: DNA testing via Polymerase Chain Reaction (PCR) and immunohistochemistry (IHC) (Poulogiannis and Arends et al., 2009).
Due to the presence of MSI-H in both types of CRC, molecular analysis and genetic testing have proven to be essential in order to identify the genetic targets associated with MMRd. Studies have shown that the altered allele size in the PCR-amplified product of DNA originating from tumors is an indication of MSI-H. Histopathology and IHC have also shown differences in the expression of \textit{MSH2}, \textit{PMS2}, \textit{MLH1}, and \textit{MSH6} in tumor cell nuclei between MSS and MSI-H. However, the loss of nuclear protein detected by IHC has cemented it as an effective method in identifying MMRd, which indicates MMR gene germline mutation carriers (Stone and Houlston et al., 2001).

The expression of \textit{MSH2}, in particular, has been studied in the ISC in order to identify stem cells influenced by MMRd. The Vilar research group has validated stem cell and non-stem cell specific genes in MMRd ISCs by assessing genes that were dysregulated between \textit{Lgr5-EGFP+} stem cells and \textit{Lgr5-EGFP-} daughter cells that were either \textit{MSH2-HET} or \textit{MSH2-KO}. A gene of interest, \textit{SPP1}, codes for a protein involved in upregulating interferon gamma and IL-12, which activate macrophages and regulate T cells, respectively. There was also an observed high level of upregulation in \textit{Spp1} in both \textit{MSH2-HET} and \textit{MSH2-KO} compared to \textit{MSH2-WT}. Additionally, there was also a positive correlation between \textit{Spp1} and \textit{Lgr5-EGFP+} stem cells, which suggested that levels of \textit{Spp1} were significantly higher in MMRd ISCs when interacting with the stem cell niche and its resident immune cells (Bommi et al., 2021). Overall, this established that \textit{Spp1} could be used as a biomarker for MMRd carcinogenesis and showed the localization of this MMRd marker in the small intestine of 8-week-old \textit{MSH2-WT}, \textit{MSH2-HET}, and \textit{MSH2-KO} mice. From this, it was observed that \textit{Spp1} colocalized with \textit{Lgr5+} cells at the base of crypts in both MMR haplo-insufficient and deficient tissues, providing...
a MMRd stem cell signature (Bommi et al., 2021). *Spp1* has been closely associated with tumor-associated macrophage (TAM) infiltration which led to the question of how macrophages interact with the stem and daughter cells within the ISC niche.

Furthermore, ISC-specific genes, such as *LGR5*, have been shown to have different expression levels depending on MMR status. *LGR5* is a gene that encodes for a receptor in the Wnt signaling pathway in the colon and its expression is almost exclusively localized at the base of the colonic crypt. The Wnt signaling pathway has an important role in the maintenance of stem cell reservoirs and the activation of their proliferation, thus controlling the rate of differentiation in the colon crypt. *LGR5* has been identified to be a marker of both normal and MMRd ISCs (Haegebarth and Clevers, 2009). The cells that express *LGR5* are the cells that have the ability to self-renew and give rise to the rest of the cells in the mucosa, showing that MMR deficiency has widespread effects within the intestine (Stoian et al., 2016).

The Vilar research group has also contributed to this field through novel research and findings from a Phase Ib, placebo-controlled, randomized clinical trial of naproxen dosage. In this trial the group analyzed whole transcriptomic changes with patient-derived organoids (PDOs) that were treated with both low (220 mg/d) and high doses (440 mg/d) of naproxen daily for 6 months. From the organoids they assessed using mRNA-seq, there were several genes involved in pathways deregulated by naproxen and downregulation in genes in the stem cell compartment and in the differentiation compartment of the ISC niche compared to placebo (Fig. 1) (Reyes-Uribe et al., 2021). In addition, pathway enrichment analysis revealed enrichment of certain signals that were involved in immune pathways, including those that activate cytokine signaling.
These expression pathways led to using \textit{in silico} deconvolution tools from the mRNA-seq data to confirm that naproxen exposure increased the activation of intestinal resident immune cells (Reyes-Uribe et al., 2021). These resident immune cells included but were not limited to T-cells, macrophages, and B-cells. Together, this data suggested that naproxen induced epithelial differentiation in the intestinal crypt via activation of the immune system because of enrichment observed in immune pathways via foldchange in mRNA-seq data comparing naproxen to placebo.

![Volcano Plot](image)

\textbf{Figure 1.} Naproxen downregulates genes of the ISC niche in PDOs. Volcano plots of differentially expressed genes (DEGs) that shows gene expression data from whole transcriptome sequencing from patients that were given placebo, low-dose naproxen, and high-dose naproxen treatment (Reyes-Uribe et al., 2021). Used with permission from copyright holder to reuse this figure.

Naproxen’s primary mechanism of action is to inhibit cyclo-oxygenase-1/2 enzyme, which reduces prostaglandin levels and attenuate the transmission of inflammation signals. By analyzing differences in gene expression pre and post exposure to the low (LD) and high doses (HD) of naproxen, it was observed that there was an overall effect of downregulation of 38 genes and 57 genes, respectively as a result. Notably, the LD and HD of naproxen exhibited differential effects within the colorectal mucosa. The LD was shown to downregulate genes involved in the cell cycle, whereas the HD downregulated genes relating to cell dynamics at the top of the colon crypt but also upregulated immune genes. This was interpreted as evidence that there are additional
mechanisms between the immune cell compartment and the stem cell niche that contribute to CRC carcinogenesis prevention (Reyes-Uribe et al., 2021).

Different systems in the ISC microenvironment have a role in affecting stem cell maintenance. The proliferation and differentiation of the ISC are part of that maintenance and are influenced by multiple cellular signals. The stem cell niche undergoes maintenance so that there is a sufficient amount of turnover to create a barrier of normal epithelial cells against any developing tumor cells (Hou et al., 2021). This barrier serves to prevent chronic viral infections and metabolic diseases from progressing (Peterson and Artis, 2014). While the immune system contains many types of cells with many different functions, immune cells have been said to and have a crucial role in maintaining the integrity of the intestine (Onyiah and Colgan, 2016). The regeneration of the intestinal cells is key to the integrity of the epithelium; therefore, the immune system specifically has been shown to have an important role in stem cell maintenance. Different types of immune cells have been shown to have different specific effects on the regeneration of the ISC niche.

Among many types of immune cells, macrophages are a major component in the stroma of the intestine (Saha et al., 2016). This has been shown by analyzing the role macrophage-derived WNT have in intestinal repair in mice after inhibition via macrophage-restricted gene ablation. It was reported that this inhibition resulted in decreased epithelial proliferation and increased radio-sensitivity of the intestine (Kabiri et al., 2014). Macrophages, being significant sources of WNTs in the colon crypt, exhibit their significant role in tissue repair and regeneration of the epithelium (Saha et al., 2016). Macrophages contribute to inflammatory injury and are involved in the coordination of
tissue repair due to their ability to acquire different phenotypes that each differ in the expression levels of surface proteins and cytokine production. Two of these phenotypes are the proinflammatory (M1) phenotype and the anti-inflammatory (M2) phenotype which can also be immune-suppressive. The M1 phenotype functions to mediate the host’s defense from microorganisms whereas the M2 phenotype expresses high levels of cytokines and other scavenger molecules that are involved in wound healing and are crucial for tissue repair (Cosin-Roger et al., 2015). Moreover, it has also been demonstrated that the M2 macrophage phenotype overexpress Wnt ligands such as Wnt1 and Wnt3a which are involved in the Wnt signaling pathway, which promotes mucosal repair (Cosin-Roger et al., 2015).

In addition, macrophages have been shown to secrete several cytokines that contribute to the maturation of the intestinal epithelium and epithelial barrier. Cytokines such as IL-6, IL-8, and IFN-gamma were produced at high levels shown by an increase in expression level in macrophage-enteroid co-culture compared to the enteroid monolayer by itself. This suggested that these cytokines were primarily secreted from macrophages rather than from the intestine-derived enteroids themselves. In addition, when macrophages were present, and therefore where the cytokines were elevated, it was shown that epithelial cell height increased in both non-differentiated (ND) and differentiated (DF) enteroid monolayers. Furthermore, the transepithelial electrical resistance (TER), which is a measurement of barrier function of epithelial cells, was also elevated suggesting increased maturation of the intestinal epithelium and a thicker physical barrier (Noel and Zachos, 2017). Therefore, as a result, it has been heavily
suggested that these secreted cytokines are involved in the role of macrophages influencing the intestinal epithelium through paracrine signaling.

The mutations that arise from LS are a result of a deficient MMR system that allows frameshift mutations to exist unchecked and cause uncontrolled cell proliferation. The key genes that are affected by the deficient MMR system are \( MSH2 \), \( MLH1 \), \( MSH6 \), and \( PMS2 \), which have been shown through molecular analysis, DNA testing, and IHC. The expression of \( MSH2 \) has been linked to the expression of \( Spp1 \) and \( LGR5 \) in the ISC. This has led to more research regarding stem cells in colonic tissue, as stem cells have the unique ability self-renew. The novel research conducted regarding naproxen has shown that NSAIDs are able to alter specific genes and pathways, including those that are related to the resident immune cells within the ISC niche. This has led to an interest in learning how immune cells are influenced by naproxen and what downstream effects an activated immune system could have on affecting CRC carcinogenesis through immune cell recruitment and immune cell secretion of cytokines.

Despite the volume of research conducted in this field, there are still gaps of knowledge that remain. The first research question of the study is focused on understanding how immune cells affect the stem cell state in the ISC niche through crosstalk in LS. Thus, I proposed using a system to detect specific biomarkers present in normal colonic mucosa before and after chronic exposure to naproxen. I utilized an \textit{in situ} hybridization assay to identify and distinguish stem cells and epithelial cells using distinct mRNA biomarkers. Through this I was able to analyze the localization and expression of these markers within tissue sections biopsied from human patients who were given naproxen as a treatment. Next, I sought to answer the question of what the
effect of this interaction looked like in living organisms. Thus, I utilized an ex vivo mouse model and immune cells to replicate the pathophysiology of the microenvironment in living mice. Finally, this study also aimed to answer what effect, if any, that the secreted factors from the immune cell compartment had on the ISC niche and stem cell maintenance.

With the approach and these strategies, I will address these missing components that will help expand the understanding of LS as it relates to MMRd. These strategies will also further promote the benefits of using naproxen as a preventive measure and as a method to counter immune cell inactivity. Based on the premises, I hypothesize that the immune cell compartment regulating the ISC niche affects its ability to renew and continue to differentiate. Enhancing the understanding of these components may have significant clinical translational potential because these components elucidate the mechanisms by which naproxen interacts with relevant immune cell types to promote both anti-inflammation and differential expression of ISC markers in the colon crypt. This could further support naproxen use as a medication for LS patients for prevention or to delay the onset of CRC and potentially provide a basis to study naproxen treatment as a way of mediating the effect of the deficiency.
Research Methodology and Design

We have used multiple methods in order to address the gap of knowledge regarding how the immune system modulates the MMRd ISC niche. These experiments required carefully selecting both human and mouse samples to limit confounding variables that could affect the interpretation of the findings. This consideration of sample selection allowed us to perform the maximum number of comparisons to provide us with more data to draw conclusions from.

Research Design

RNAsecope Experiment Design

For the proposed studies a multiplex nucleic acid *in situ* hybridization technology was used to selectively identify and amplify mRNA signal from specific genes while reducing background noise. This multiplex *in situ* hybridization RNAsecope assay (Cat #323100) was performed on Formalin-Fixed Paraffin-Embedded (FFPE) tissue sections of three MMRp human patient biopsies of the rectosigmoid colon that were fixated on SuperFrost Plus slides. These biopsies of colon originated from patients at MDACC and were collected from patients who had not been diagnosed with LS. Therefore, the tissue sections identified as MMRp tissue sections conceptually represent the population without a germline mutation and without the associated increased susceptibility to cancer. Information about these patients that was collected included age, race, gender, cancer history, and cancer type. Conversely, the colon biopsies collected from patients who had been diagnosed with LS were identified as MMRd. Each target probe was individually hybridized and assigned a different fluorescent detector molecule in order to...
distinguish the signals. This same methodology of determining MMR status was used for the sections on the tissue microarray (TMA) for the quantification of the MMRd tissue sections. The staining process followed the Multiplex RNAscope assay protocol (ACDBio, Newark, CA), and the slides were mounted with Prolong Diamond Antifade mountant (ThermoFisher, Dallas, TX). The slides were then stored at 4°C for at least 24 hours but no longer than 96 hours before being scanned, utilizing the VectraPolaris 1.0 Automated Quantitative Pathology imaging system. Each channel was optimized individually within the Phenochart imaging software and slide viewer to ensure that the least amount of autofluorescence carried over into the imaging analysis.

This RNAscope assay is an *in situ* hybridization assay that uses 20 target-specific double Z-shaped probes that target a 1-kb region on each RNA molecule. This novel design improves signal-to-noise ratio and allows for selective amplification of target-specific signals, thereby only hybridizing with specific target molecules. Through a cascade of hybridization events, pre-amplifiers hybridize to the binding site on each double Z probe which allows the subsequent binding of amplifiers that amplify the detection of target RNA molecules. Labeled probes containing fluorescent molecules are then able to bind to the amplifier, allowing for fluorescent detection. The mRNA biomarkers detected were *LGR5* (Cat #311021), *ASCL2* (Cat #311011-C2), and *EPCAM* (Cat #310281-C3) mRNA for visual analysis through the Multiplex RNAscope assay which is capable of simultaneously detecting up to 3 biomarkers. In order to ensure that the three fluorescent excitation and emission wavelengths from the molecules did not overlap, they were chosen to be sufficiently distant from one another on the visible light wavelength spectrum.
We then run several controls for this assay to ensure that the hybridization assay was optimized for tissue type of the samples. The genes, *Polr2a* (Cat #320861) and *UBC* (Cat #320861), were used as positive control markers stained by Opal 520 and 620, respectively. The bacterial *DapB* gene (Cat #320871) was used as a negative control marker stained by Opal 570 (Fig. 2). These positive controls were first tested on HeLa cell pellets (Cat #310045) to establish the sensitivity of those genes to DAPI-stained cells and then subsequently tested on the MMRp tissue sections. The positive control genes were chosen due to the ubiquitous expression across all types of tissue. The negative control, *DapB*, was chosen because it is expressed in bacterial cells and lacks expression in human cells.

![Figure 2. Positive and negative controls for RNAscope assay.](image)

The patient samples for the FFPE tissue sections were collected from the rectosigmoid normal colon mucosa tissue sections to establish a baseline density and distribution of these markers. The number of samples was chosen to guarantee there were sufficient amounts of tissue and colon crypts to analyze. These samples were
collected from 5 trial participants, all 18 years of age or older. 3 of the patients were male and 2 were female with 2 of the 5 total having a history of cancer. Of the 2 patients with a cancer history, 1 had been diagnosed with prostate cancer and the other had been diagnosed with skin cancer (Table 1). These samples were collected from participants without a germline mutation to represent the average risk of acquiring CRC in the population. The trial participants were required to consent and characterized as having intact portions of the distal and rectosigmoid colon for sample collection to occur.

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Table 1. FFPE tissue sections from control patient samples. List of control patient samples (n=15) and their designation regarding the study ID, age, gender, race, ethnicity, history of cancer, and cancer type.

**Mouse Model Experiment Design**

The macrophage conditioned media was collected from RAW 264.7 mouse macrophages (ATCC, Houston, TX) that were cultured in T-75 flasks with Corning DMEM media with L-Glutamine, 4.5g/L Glucose, and Sodium Pyruvate additives (Fisher Scientific, MT10013CV). The macrophages adhered to the flask and once the cells had reached 70% confluency, the media was removed the media and collected it in 15mL conical tubes. Next, the media was filtered in 0.8 μm filters (NovoLab, A06228) to remove
anything that was an extracellular matrix component so that the media only contained factors secreted from the macrophage cells. Once the media was filtered, 4 different concentrations of the conditioned media were prepared: 0% as the control, 10%, 25%, and 50% concentrations. The concentrations were prepared with L-WRN media derived from stem cells without any growth factors added and was generated from L-WRN_WNT sorted cells.

A novel mouse model was used to replicate the conditions of LS in mice to better understand the MMRd ISC niche. This allowed the analysis of changes in the genetic profile of MMRd cells in response to factors secreted by a simulated immune cell compartment using ex vivo mouse-derived organoid models. Thus, this setup allowed observations of these specific effects on target genes of interest. The colons from a total of 6 mice between 6 and 8 weeks of age were harvested and were developed into an organoid mouse line from each individual mouse (Fig. 3).

Figure 3. MMRd mouse model organoids. Image of organoids derived from Msh2<sup>LoxP/LoxP;</sup>Villin-Cre;TGFβRII<sup>Hu</sup> mouse line at 3<sup>rd</sup> passage level before harvesting taken under a light microscope.
These colons were harvested using the isolation of mouse intestinal crypts protocol and were grown using the Organoids Culture from Isolated Mouse Intestinal Crypts protocol (Stem Cell Technologies).

Out of these 6 mice, 3 of which were of the \textit{Msh2}\textsuperscript{LoxP/LoxP};\textit{Villin-Cre};TGF\betaRII\textsuperscript{Hu} line and the other 3 were of the wildtype C57BL/6 line. In the \textit{Msh2}\textsuperscript{LoxP/LoxP};\textit{Villin-Cre};TGF\betaRII\textsuperscript{Hu} line, the \textit{Msh2} \textit{loxP} allele was crossed with the \textit{Villin-Cre} transgene to result in an intestinal specific \textit{Msh2} knockout. In this knockout the \textit{Msh2} allele was excised by Cre-recombinase in tissue that expresses \textit{Villin}. The lineage from these mice express the \textit{Msh2} knockout in the intestine (Kortüm et al., 2015). These mice that expressed the \textit{Msh2} knockout had intestinal epithelium specific inactivation of MMR. The C57BL/6 line is a common inbred strain of mouse which represented the average mouse in the population which does not express the \textit{Msh2} knockout.

These organoids from both mouse lines were cultured and given L-WRN stem cell-derived growth media until passage number 3 to ensure that the organoids were growing normally without infection in the 3D culture matrigel. As outlined in the Passing Mouse Intestinal Organoids protocol, passaging occurred when seeding density was at 200 organoids per well (Stem Cell Technologies). This was determined using a light microscope to visualize the organoid cultures.

Once these organoids had reached the indicated passage level, they were treated with these indicated concentrations of macrophage conditioned media on 24-well plates. This was organized so that there was a total of 8 wells of organoids treated with the same media for each of the 3 biological replicates for both mouse genotypes. The organoids
were incubated in this media for 48 hours before harvesting the cells. Once the cells were harvested, a TaqMan Cell-to-CT kit (ThermoFisher, A35374) was utilized to produce the sufficient amount of cDNA necessary to analyze the gene expression for the genes *LGR5*, *ASCL2*, and *Villin*.

These organoids were also grown on separate 24-well plates; 3 wells were treated with each concentration of conditioned media for a total of 12 wells. These plated organoids were harvested and used to measure cell viability using the Cell Titer Glo 3D assay (Promega, G9691). In order to measure cell viability of these organoids, this assay was used to detect the presence of NanoLuc luciferase, a mutant Ultra-Glo thermostable recombinant luciferase. Its presence indicated that cells within the structure were alive and viable whereas a lack of NanoLuc signal indicated that the cells were not living and therefore not viable.

For the conditioned media analysis, 2 duplicates of each of the three types of samples were sent to the Mass Spectrometry Baylor Proteomics Core: 100% conditioned media, 50% conditioned media, and 0% conditioned media. The proteomics core conducted mass spectrometry (MS) on the pooled peptide fractions and from that analysis, data analysis including names of secreted peptides present in that media, quality control (QC) metric data, principal component analysis (PCA), clustering (hierarchical, k-means) data, differential expression profiles (volcano plots and statistics), gene-set enrichment analysis (GSEA), and annotations of relevant protein categories of interest was obtained. Additional pair-wised comparisons were performed between all three of the sample types and additional GSEA to highlight areas I was specifically interested in observing.
**Explanation of Methodology**

**Tissue Microarray**

On the TMA generated from the Naproxen Trial that was utilized to analyze the RNAscope assay to detect mRNA expression, there were a total of 15 patients where colon samples were collected from and analyzed. The trial participants were required to be characterized as having intact portions of the distal and rectosigmoid colon for sample collection to occur and provided written informed consent for biopsies of normal mucosa to be collected (Fig. 4). The samples on this TMA from participants represented the population with a germline mutation that had been diagnosed with LS.

![Image](image.png)

**Figure 4. Naproxen Trial TMA.** Hematoxylin & Eosin (H&E) stain of distal and rectosigmoid colon FFPE samples on the TMA used for RNAscope.

These patients were all of 18 years of age or older and contained a pathogenic or likely pathogenic mutation of one of the MMR genes. The distribution for the treatment that the patients received was 5 placebo, 6 LD, and 4 HD. 7 of the patients were male
and 8 were female within the ages of 30 and 82. 9 of the 15 patients had a history of cancer. Each of the 15 patients were organized based on the type of treatment in the naproxen trial: placebo, LD, or HD naproxen (Table 2). The FFPE normal colon mucosa samples were collected using the Protocol for Formalin-Fixed Paraffin-Embedded (FFPE) Tissue (BioSearch Technologies).
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Table 2. Population information from Naproxen trial participants. List of patient samples (n=15) and their designation regarding the site of biopsy collection, age, gender, race, ethnicity, treatment type, MMR gene mutated, DNA change, protein change, and history of cancer.
**Mouse-Derived Organoids**

The mouse-derived organoid experiments were organized where 3 mice from the \( Msh2^{\text{LoxP/LoxP; Villin-Cre; TGF\(\beta\)RIIHu} \) genotype and 3 mice from the C57BL/6 genotype were grown between 6 and 8 weeks of age to be sacrificed for their colons to be harvested and used to generate organoids. The cell viability data was collected from these organoids using a luminescence microplate reader (BioTek Synergy HTX). Gene expression data for each of the four different types of conditioned media treatment for both mouse genotypes was collected via quantitative Polymerase Chain Reaction (qPCR) on a 7900HT Fast Real-Time qPCR Thermal Cycler (Applied Biosystems) using the universal SYBR Green qPCR protocol under standard cycling parameters. The primer used as the housekeeping gene was \( GAPDH \) and the experimental genes were \( LGR5, \ ASCL2 \), and \( Villin \). The gene expression was calculated using the double delta Ct method for relative quantification.

**inForm Automated Image Analysis**

In addition to using the VectraPolaris 1.0 Automated Quantitative Pathology imaging system, the inForm software was also used for several steps of the process leading up to the quantification of mRNA transcripts per cell. After each core of tissue was selected from the scans of the individual patient sample slides or the TMA, an individual image file was created. These signal channels were then isolated and unmixed to minimize any overlap in signal channels and to remove as much autofluorescence as possible. The three fluorescent molecules to detect \( LGR5, \ ASCL2 \), and \( EPCAM \) were Opal 620, Opal 690, and Opal 520, respectively.
**Bioinformatics**

Part of the analysis for the conditioned media included utilizing the Database for Annotation, Visualization, and Integrated Discovery (DAVID) to sort through the proteomic profile and organize categorical associations with different pathways based on molecular function. For the pathway analysis I utilized the KEGG_PATHWAY and GO_TERM ontologies to represent the data collected.

**Quantitative Measures**

The biomarker signal was quantified in the imaging software, Visiopharm, to detect the number of cells containing the specific markers and to detect the number of individual signals. The regions of interest (ROIs) were manually selected by marking colon crypts and the selection of the ROIs was approved by a pathologist in order to ensure that the ROI selection was representative of the entire sample of normal colon mucosa.

Utilizing the Visiopharm software, each marker was given a different threshold of detection to ensure that the quantification was optimized for each type of signal. Visiopharm also enabled the identification of individual cells within the ROIs which made it possible to determine if the signal was present inside or outside of a cell. This allowed the quantification to distinguish the number of signals detected based on relative location. Furthermore, the number of cells and the area for each ROI were measured and recorded to use for normalization in the quantification analysis. In order to achieve this, the Visiopharm software also used a Deep Learning AI-based software in order to detect and identify nuclei based on the intensity of the DAPI counterstain. The program used changes in pixel intensity of the image to identify boundaries between the cell nuclei in
order to distinguish one cell from another for counting the number of cells. In order to present the data, two different methods of ROI selection were used.

The first method of analysis consisted of choosing an ROI on each patient tissue sample so that each ROI contained four colon crypts. In this method of analysis, each ROI contained the same number of colon crypts from every patient tissue sample. With this methodological approach, the selected ROIs that contained crypts were transversally cut so that most of the crypt or the entire length of the crypt was able to be visualized (Fig. 5). In this analysis, the numeric values of mRNA transcripts per cell (subcellular spots) were then normalized to the total area of the ROI selected in order to take into account variation in the size the total area of the ROI and also the size of crypt. These steps enabled the analysis of changes in gene expression in the colon crypt cells both with and without exposure to naproxen on a molecular level.

Figure 5. First method of analysis for ROI selection in Visiopharm. Image of the Visiopharm AI software identifying cell nuclei based on its Deep Learning AI-based protocol within a single ROI (outlined in red) manually selected to include 4 full colon crypts from patient tissue samples.
The second method of analysis consisted of selecting the base region of individual crypts in order to focus the quantification on the stem cell markers which reside at the base region of the crypt. This selection was performed with the guidance of a pathologist with the intention of reducing the effect that large ROIs had on diluting the density of the mRNA biomarkers due to the size variation of the colonic crypts. In the analysis, due to varying sizes of the base regions of the crypts, the mRNA density data generated was normalized by the number of cells identified within each ROI. The identification of the cells was performed through Visiopharm by detecting the nuclei of the cells within the crypts. With this methodological approach, each individual colon crypt was considered its own ROI. The base regions of these ROIs were identified and selected using their proximity to the muscularis mucosae layer of the tissue to ensure that the selected cells were actually at the base of the crypt for the analysis instead of cells that appeared to be at the base (Fig. 6). The number of ROIs selected per patient sample was variable and the analysis consisted of normalizing to the total area selected.

![Figure 6. Second method of analysis for ROI selection in Visiopharm. Image of the Visiopharm AI software identifying cell nuclei based on its Deep Learning AI-based protocol within 4 individual ROIs manually selected to contain the bases of 4 colon crypts from patient tissue samples.](image-url)
The analysis of the macrophage conditioned media was performed by loading 5% of conditioned media sample on an SDS-PAGE gel where an in-gel digest of proteins was performed. The pooled peptide fractions were then sequenced using a mass spectrometer against the NCBI RefSeq database. gpGrouper was then used to perform gene-centric protein identification and quantification.

**Statistical Measures**

The GraphPad statistical software was utilized to determine if there was statistical significance for the data collected. Grouping the quantified and normalized RNAscope signal detection data, t-tests were run between groups based on tissue type: control or LS. T-tests were also run between groups based on naproxen exposure for the LS patient samples.

In order to illustrate the magnitude of significant difference of cell viability between the control (0%) and the 10%, 25%, and 50% treatment concentrations of macrophage conditioned media, t-tests were run between those groups. These data sets for gene expression were generated in triplicates for each of the biological replicates.

The magnitude of significant difference of gene expression between the control (0%) and the 10%, 25%, and 50% treatment concentrations of macrophage conditioned media was also illustrated through running t-tests between groups. These data sets for gene expression were once again generated in triplicates for each of the biological replicates.

The macrophage conditioned media analysis was performed through multiple methods which consisted of: Quality Control, Principal Component Analysis (PCA),
Clustering (hierarchical, k-means), Differential Expression, Gene Set Enrichment Analysis (GSEA), and Annotation of Protein Categories. For differential analysis and GSEA, the pAdj value cutoff was set to 0.05 and the LogFC cutoff was set to 2.

**Delimitations and Limitations**

In order to narrow this study to the scope of our hypothesis, only a limited number of genes that were believed to be representative of the populations of cell types were observed. In addition, only the effects of conditioned media from macrophages were observed due to existing research that supported macrophages having an important role in influencing the maintenance of the ISC niche.

There were several limitations encountered within this study. The primary limitation was the number of samples available to utilize for the RNAscope imaging. Many of the tissue cores were either fixated poorly where the image was blurry after scanning, did not contain a sufficient amount of tissue to analyze, or the tissue did not contain a sufficient number of colon crypts to analyze. Due to this limitation, it was not possible to include the full number of patient samples that were marked as available on the TMA. As a result, the LD and HD naproxen treatments were grouped into one category to compare differences between placebo and naproxen exposure. Due to the variation in number of colon crypts, there were only a few number ROIs able to be selected across all of the patient samples. This limitation led to the selection of one ROI for every available patient sample to ensure that the distribution between patient samples was even for comparison between groups based on naproxen exposure.

**Ethical Considerations**
As this study used patient samples and required the sacrifice of several mice, there were several considerations taken under IACUC protocol #00000469-RN02 (Characterization of the Genomic Landscape of Intestinal Adenomas in Mouse Models of FAP) and protocol #00000488-RN02 (Amendment 7 for IACUC Study #00000469-RN02) in order to ensure the research methodology was ethical. The patient samples that were used for RNAscope had already been collected through the Naproxn Trial (NCT02052908, MDACC IRB# 2013-0698), a phase Ib double-blind trial in an ethical manner where the identity of the patients remained confidential from January 2014 to October 2017. The trial participants were first identified then recruited from the following research registries: clinical cancer genetics and gastroenterology. The study was sponsored by the National Cancer Institute (NCI) and ethical approval was granted by the Institutional Review Board (IRB) at The University of Texas MD Anderson Cancer Center (MDACC) on 01/27/2014 and at Dana Farber/Brigham and Women’s Cancer Center, The University of Michigan, and Huntsman Cancer Institute.
Research Findings

Over the conducted research experiments, many findings suggest that the immune cell secretome was a factor in modulating the ISC niche. The findings support the central hypothesis that the immune cell compartment regulates the MMRd ISC niche. The findings also illustrate this through more than one methodology to increase the validity and confidence in our interpretations and conclusions.

RNAoscope Density

Utilizing the RNAscope in situ hybridization methodology, the distribution of the ISC and epithelial cell markers within the colon crypt was visualized. EPCAM, the epithelial marker shown by Opal 520 (green), was observed to be highly expressed in the middle and top regions of the colonic crypts, and the stem cell markers, LGR5 and ASCL2 shown by Opal 690 (orange) and Opal 620 (red) respectively, localized towards the lower region (base) of the colonic crypts (Fig. 7).

![Figure 7. Distribution of biomarkers within colon crypt. Visualization of LGR5, ASCL2, and EPCAM markers in section of rectosigmoid colon biopsied from the control normal mucosa stained by Opal 690, Opal 620, and Opal 520, respectively.](image)
First, the density and distribution of stem cell markers was assessed between sporadic samples collected from participants (control) and LS samples collected from participants. These control samples represented the population without a germline mutation that increased susceptibility to cancer and was used for the control group of samples. This type of analysis was intended to determine the difference between participants who had the average risk of acquiring CRC in the population and LS participants. When quantifying the density of mRNA transcripts per cell (subcellular spots) in normal mucosa, there was a significant difference between the control and LS cases with both stem cell markers: LGR5 and ASCL2 shown by a reduction in LS cases (unpaired t test: p<0.0001; p<0.0001) compared to control. This suggests that MMRd does influence the density of stem cells within the ISC niche with a reduction in that cell population. The density of these stem cell markers was shown to be reduced in the tissue containing germline mutations in MMR genes contributing to MMRd (Fig. 8).

**Figure 8. Reduced stem cell marker density in LS cases.** Quantification of ASCL2 and LGR5 markers following RNAscope analysis of Sporadic (control) (n=5) and Lynch Syndrome tissue sections (n=13) and by area of the ROIs selected. Each data point on the graph represents the number of mRNA transcripts per cell (subcellular spots) within the ROIs selected from each patient sample.
This analysis of density and distribution of stem cell and differentiated cell markers from the effect of naproxen in MMRd normal mucosa patient samples was assessed and normalized using the methods previously introduced (Quantitative Measures). In the first method of analysis, the density of areas containing the entire colon crypt was analyzed and it was observed that there was a distinguishing trend between the mRNA biomarkers. As expected, there was no significance difference in expression of any of the markers (LGR5, ASCL2, and EPCAM) between samples taken on day 1 before the patients began taking Naproxen and after daily treatment with placebo for 6 months (unpaired t test: p=0.2690; p=0.7792; p = 0.9475). However, when naproxen was given at either LD or HD (220 or 440 mg/d), there was a significant increase in mRNA transcript detection (subcellular spots) of both stem cell markers, LGR5 and ASCL2 within the ROIs (unpaired t test: p=0.0168; p=0.0120) (Fig. 9).

Figure 9. Increased LGR5 and ASCL2 marker densities following Naproxen exposure in LS cases. Quantification of LGR5, ASCL2, and EPCAM ISC markers following RNAscope analysis of LS colon tissue sections before and after exposure to 6 months of placebo and Naproxen (n=20). The ROIs were manually selected to each be comprised of 4 colon crypts. Each data point on the graph represents the number of mRNA transcripts per cell (subcellular spots) within the ROIs selected from each patient sample.
In the second method of analysis, the changes in density were analyzed by observing what occurred at the base of the colon crypt structure by selecting crypts to be the ROIs from the patient samples. The patient samples were grouped into 4 categories: Pre Placebo, Post Placebo, Pre Naproxen, and Post Naproxen. The number of ROIs were selected based on the quality and size of tissue in the patient sample. In the Pre Placebo cases there were a total of 9 ROIs that were selected and analyzed from 3 patient samples. In the Post Placebo cases there were a total of 11 ROIs that were selected and analyzed from 3 patient samples. In the Pre Naproxen cases there were total of 39 ROIs that were selected and analyzed from 9 patient samples. In the Post Naproxen cases there were a total of 31 ROIs that were selected and analyzed from 7 patient samples (Table 3). In this case, there was no significant change in mRNA transcripts per cell (subcellular spots) between the two stem cell markers (LGR5 and ASCL2) before and after treatment with LD or HD naproxen for 6 months (unpaired t test: p=0.2970; p=0.6930). However, there was a significant difference in EPCAM (unpaired t test: p=0.0075) (Fig. 10). Specifically, this data showed a significant increase in the density of EPCAM after 6 months of naproxen treatment.
### Table 3. ROIs from patient samples of the Naproxen Trial

List of patient samples from the TMA of the Naproxen Trial. From each of the patient samples (Sample IDs) a number of ROIs were identified and selected for quantification analysis.

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Viability of Mouse-Derived Organoids

The next findings consisted of examining the effect of the conditioned media collected from RAW 264.7 mouse macrophages on cell viability of passage 3 wildtype and LS mouse-derived organoids. The data showed that increasing the concentration of macrophage conditioned media, increased the cell viability of the wildtype mouse organoids (n=3) (Fig. 11A). This increase was consistent from each of the four different concentrations of media (0%, 10%, 25%, and 50%). However, increasing the concentration of macrophage conditioned media only marginally increased the cell viability of LS mouse organoids with respect to the control compared to the WT organoids (n=3) (Fig. 11B). There was an initial increase in viability between the 0% and 10% concentration with a gradual decrease in viability from 10% to 50%.
The next finding consisted of examining the effect of the macrophage conditioned media on the gene expression of three genes of interest: LGR5, ASCL2, and Villin. Comparing the gene expression when LS mouse-derived organoids were exposed to the L-WRN stem cell growth media and the 50% macrophage conditioned media, the two stem cell biomarkers, LGR5 and ASCL2, were downregulated significantly in the 50% macrophage conditioned media. However, the gene Villin, which is expressed in intestinal epithelial cells, was significantly upregulated in LS mouse-derived organoids (Fig. 12). This decrease was not shown in the wildtype mice with no significant difference in either of the stem cell markers. However, there was a significant increase in Villin expression.
Figure 12. Macrophage conditioned media incubation of mouse-derived organoids influences gene expression. Fold gene expression from qPCR of LGR5 (top), ASCL2 (middle), Villin (bottom) after 48-hour incubation with control L-WRN media (black) and 50% (green) concentration of RAW 264.7 macrophage conditioned media. Organoids were derived from C57BL/6 wildtype mice (n=3) and Msh2$^{LoxP/LoxP}$;Villin-Cre;TGFβRII$^{Hu}$ mice (n=3).
Analysis of Secretome Profiling

After analyzing the effect of the different conditioned media concentrations on the fold gene expression of relevant markers for stem cells and epithelial cells, the next step was to analyze the components of the conditioned media. The analysis of the conditioned media illustrated important differences in the proteomic make-up between the control L-WRN stem cell growth media and the macrophage conditioned media. Differential analysis was performed on three different concentrations of media with two replicates for each: L-WRN stem cell growth media, 50% macrophage conditioned media with 50% L-WRN media, and 100% concentration of macrophage conditioned media. A total of 763 gene sequences associated with proteins were found to be present in common among the 3 media mixtures. A distinct difference was observed in the proteomic profile between the L-WRN stem cell growth media (cultureStemCell) and the 100% concentration of macrophage conditioned media (cultureMacrophage). There were a number of significant genes found based on the Benjamini-Hochberg (BH)-adjusted P-value < 0.05 and log2FC > 2 value cutoffs including Gpi1, Txn1, PPla,
Ccl4, and Lgals3 which were all elevated in cultureMacrophage whereas Alb, A2m, Itih2, Lum, and Ahsg were elevated in cultureStemCell (Fig. 13).

The GSEA of the L-WRN stem cell growth media and the 100% concentration of macrophage conditioned media showed different enriched gene ontology pathways. The molecular function from the gene ontology included but was not limited to enrichment of cytokine activity, nucleic acid binding, protein dimerization, and transcription regulator activity in the 100% concentration of macrophage conditioned media compared to L-WRN stem cell growth media (Fig. 14). This enrichment in these notable activities were a result of the factors secreted from macrophages in the conditioned media which would not have been present in the stem cell growth media. These activities also show that the secretome from the macrophages are associated with different levels of activity including cytokine activity, protein dimerization activity, heparin binding, and growth factor binding. The factors that contribute to these specific activities were significantly elevated (pAdj cutoff = 0.05) in cultureMacrophage.
Out of all the genes identified in the GSEA there were 763 identified genes that had been categorized as being secreted proteins by the Baylor Proteomics Core. As a result of the set up for the Viability of Mouse-Derived Organoids experiment with no cell-to-cell contact between the mouse organoids and the RAW 264.7 mouse macrophages, the sequences associated with secreted proteins were most relevant for the subsequent analyses. The pair-wise comparisons illustrated the differences in the proteomic profiles based the number of sequences identified through the analysis (Fig. 15). The proteomic profiles from the macrophage and mixture samples compared to the stem cell samples reflect a number of proteins that were exclusively found in the macrophage or mixture samples.
Figure 15. Proteomic profiles of conditioned media samples. Heatmap from the union of the top 30 significant genes from 3 possible pair-wised comparisons for all 6 samples: 2 macrophage, 2 mixture, and 2 stem cell. The gene names marked in red represent the genes that are the most significantly different across the 3 comparisons. The numbers correlate to the number of sequences identified. The z-score indicates the values in comparison to the mean value (red denotes higher than the mean and blue denotes lower than the mean).
After identifying the secreted proteins present exclusively in the macrophage samples of conditioned media, the official gene symbols of the 444 genes elevated in macrophage conditioned media (log2FC > 1) were run through Database for Annotation, Visualization, and Integrated Discovery (DAVID) to determine the types of pathways that these secreted factors were involved in. The Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) database collections were utilized in this analysis (Table 4 and Table 5). The KEGG and GO databases identified several pathways that were associated with the secreted proteins present exclusively in the macrophage samples of conditioned media. Signaling pathways including HIF-1, IL-17, and Hippo in addition to binding activity including Hydrolase, ATP, and Oxidoreductase were upregulated in the macrophage conditioned media samples (FC>1).
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Table 4. Pathways upregulated in KEGG database of macrophage conditioned media samples. List of KEGG pathways associated with the greatest number of genes (FC >1) upregulated in the macrophage conditioned media samples.
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Table 5. Gene Ontology of macrophage conditioned media samples. List of GO pathways associated with the greatest number of genes (FC >1) upregulated in the macrophage conditioned media samples.
Conclusion

Discussion of Research Findings

In the quantification of the mRNA molecules for each of the markers, our finding of significant difference between stem cell marker density (LGR5 and ASCL2) in control and LS normal colon mucosa suggests that deficiency of the MMR system reduces the density of stem cells in the ISC niche. This reduction also suggests that MMRd may be capable of influencing the density of other types of cells within the colonic crypt because every differentiated cell arises from their progenitor stem cells.

When the naproxen trial patients were treated with a daily dose, either LD or HD, for 6 months, our finding of significant difference in the stem cell marker density compared to placebo suggests two things. Our two different methods of ROI selection for this data allowed us to focus on both the changes at the base of the crypt and the changes throughout the entire colonic crypt. When quantifying the changes in density throughout the entire crypt, our observation that naproxen exposure influenced the stem cell marker density suggests a potential increase in stem cell differentiation. However, our second methodology which only analyzed the base of the colonic crypt, showed no significant difference in the stem cell marker density. Pairing these two methodologies together suggests that changes in stem cell marker density occurred higher up in the colonic crypt structure. Our hypothesis that the effect of naproxen and thereby potentially immune cell secretome induced differentiation was supported by the finding of an increase in stem cell density beyond the base of the colonic crypt.
These changes in *LGR5* and *ASCL2* density, however, were not reflected in the density of *EPCAM*. The significant increase in *EPCAM* density within the base of the crypt but not the crypt overall in the LS mouse model suggested that treatment with naproxen only influenced *EPCAM* density within the base of the crypt. As the base of the crypt is comprised of stem cells, this could have an implication for naproxen and potentially immune cell secretome inducing differentiation in this region of the crypt.

Another novel finding was the change in cell viability based on concentration of macrophage conditioned media between wildtype and LS mouse-derived organoids. This suggested that the ISC niche in LS mouse-derived organoids has a significantly different response to interaction with macrophages than the ISC niche in WT-derived mouse organoids. The large increase in cell viability of the WT organoids at higher concentrations of macrophage conditioned media suggests an inducement of cellular proliferation. Conversely in the LS mouse-derived organoids, the response was substantially different, showing an initial increase from 0% to 10% concentration, followed by a gradual decrease in viability as the concentration of macrophage conditioned media continued to increase from 10% to 25% to 50%. The decrease in the cell viability in the LS organoids beyond the 10% conditioned media concentration could be explained by cells in the ISC niche having a negative feedback response in proliferation to the signals secreted by the macrophages beyond the 10% concentration level of saturation. This decrease in cell viability in the LS organoids could also be explained by density-dependent inhibition. In density-dependent inhibition, cell growth is regulated by the density of cells in that environment where proliferation ceases when a cell density threshold has been reached. In the context of the LS and WT organoids
experiencing this phenomenon, proliferation may have ceased at a lower concentration of conditioned media in the LS organoids compared to cells in the WT organoids due to a lower cell density threshold in the LS organoid cells.

Our research findings from the cell viability data are reflected in our findings of changes in gene expression. The decrease in *LGR5* and *ASCL2* expression between the 0% and 50% macrophage conditioned media concentrations and an increase in *Villin*, support our previous interpretation of increased differentiation. At the 50% concentration, stem cell marker expression was reduced whereas epithelial marker expression was increased. The reduced stem cell marker expression led us to the conclusion that more of the stem cells within the organoids had differentiated into other types of cells within the colonic crypt and in the process, lost their stem cell identity as supported by the reduced expression level. This reduced stem cell expression paired with the decrease in cell viability in the LS mouse model reinforced our interpretation for both sets of data. The reason for the expression of the stem cell markers not showing an increase despite the cell viability data indicating increased cell proliferation could be explained by other cell types within the ISC niche. The ISC niche is also comprised of Paneth cells, enteroendocrine cells, and transit-amplifying cells which could have contributed to the increase in cell viability that our data showed. Our experiments did not measure changes in expression for markers specifically associated with these cell types. Rather, our experiments measured changes in *Villin* which is expressed across intestinal epithelial cells. An experimental method that could explain the lack of increased expression of stem cell markers would consist of assessing the markers that identify those cell types. To identify and measure the expression of enteroendocrine cells, Cld4, a selective
marker localized to the cell surface, could be used. In a similar nature, Fzd5, a receptor for Wnt signaling proteins, and EphB2, a receptor that promotes intestinal epithelium proliferation, could be used to identify and measure Paneth cell expression. Furthermore, expression of transit amplifying cells could be measured by observing RRM2, TK1, and CDC20 markers.

In addition to different cell types within the ISC niche, macrophages also exhibit variability regarding their polarization. Macrophages can either be classically activated (M1) with a pro-inflammatory role, alternatively activated (M2) with an anti-inflammatory role, or along the spectrum between those two poles where they can exhibit characteristics of both. Macrophages can be classically activated by lipopolysaccharide (LPS) and IFN-λ and can be alternatively activated by IL-4 and IL-13. In terms of colorectal cancer, the M1 phenotype is associated with preventing tumor growth whereas the M2 phenotype is associated with promoting tumor growth. M1 macrophages carry out their pro-inflammatory role by initiating an immune response through secreting cytokines such as IL-6, IL-12, and IL-18, by producing nitric oxide (NO) and reactive oxygen intermediates to protect the environment from foreign pathogens, and by performing phagocytosis through identifying antigens on bacteria. M2 macrophages carry out their anti-inflammatory role by secreting cytokines such as IL-10 and TGF-β which suppress immune responses, reduce inflammation, and promote tissue repair.

In connection to our results, we observed an increase in cellular proliferation in the WT organoids and initially in the LS organoids. This could suggest that the macrophages in the conditioned media could have been more classically activated than alternatively
activated because cells proliferate in response to inflammatory signals, which M1 polarized macrophages secrete.

In the analysis of the 3 samples of conditioned media, the differential expression data clearly showed the difference in the factors detected between the macrophage and the mixture samples compared to the stem cell samples. This reinforced our interpretation that it was the secretome from the macrophage that was responsible for the changes in cell viability and gene expression. The molecular function GSEA data also support our interpretation that the signaling occurring between macrophages and ISCs was paracrine in signaling as our experiment setup with the conditioned media incubation made any possible cell-to-cell direct interaction impossible. This data also suggested that numerous pathways involved in the changes I observed with cell viability and gene expression contain molecular mechanisms such as protein dimerization, ubiquitin-like protein ligase binding, and transcription regulator activity.

The heatmap analysis was crucial in showing the count of distinct peptide sequences of the genes with the highest z-scores between the different types of samples. While there were some peptide sequences that overlapped between the macrophage samples and the stem cell samples, there were many peptide sequences such as Txn1, Eef1a1, and Hspa8 that were substantially upregulated in the macrophage and mixture samples. Analyzing all of the genes upregulated in the macrophage samples with a FC > 1 was an illuminating step toward looking at a bigger picture of how incubation with macrophage secretome contributed to our results. Notably, the Hippo signaling pathway is responsible for regulating cell proliferation and apoptosis. These were suggested to be relevant processes considering the cell viability and gene expression data of stem cells.
markers significantly changed upon conditioned media incubation. Upregulated factors such as ERK, Grb2, uPa, associated with proteoglycans in cancer, are also known to be involved in angiogenesis and cellular proliferation.

**Comparison to Other Findings of Prior Research**

Our lab had previously conducted research using RNA sequencing data in MMRd cells between the naproxen and placebo groups in LS patients. In this data, there were no significant changes between pre and post treatment in *LGR5* or *ASCL2* through RNA sequencing (Bommi et al., 2021). The approach that I utilized with RNAscope showed the genes being upregulated when looking at the entire crypt in a selected ROI. I believe this draws attention to our findings that observed changes in density across the cut sections of colonic normal mucosa.

**Implications**

These findings led us to question how immune cells that expressed these upregulated immune genes interacted with the colonic crypt. They also lead to more questions about how that interaction could affect prevention in LS patients. Knowing the factors that contribute to the changes in cellular proliferation, angiogenesis and pathways pertaining to gap junctions and IL-17, could be useful in determining the most relevant mechanisms for how macrophages regulate cells within the ISC niche.

**Suggestions for Use in Further Research**
Further research is still required to fully understand the full effect of immune cells on the ISC niche. In addition to the markers used in our experiments (LGR5, ASCL2, and EPCAM), other markers could be used in order to measure changes in gene expression within other cell types in the ISC niche and paint a clearer picture of how those cells respond to immune activation. Some of these could include markers for Paneth cells and enteroendocrine cells such as, Fzd5 and Cld14, respectively. Measuring the gene expression of these markers would clarify how each cell type overall contributes to our findings in cell viability. Furthermore, using markers that more specifically and exclusively target cell populations within the different regions of the ISC niche would also further distinguish cells apart that were marked by EPCAM in our experiments. This, for example could help distinguish enteroendocrine cells from enterocytes.

As a result of our experiments using conditioned media from RAW 264.7 macrophages which were not experimentally polarized to be pro-inflammatory (M1) or anti-inflammatory (M2), our experiments did not show the effects that fully polarized macrophages could have on the gene expression of cells in the ISC niche in mice. Future experiments using experimentally polarized M1 and M2 macrophages by treatment inducement with IFN-γ and IL-13, respectively, to generate conditioned media could provide clarity on whether there are differences in gene expression based on the pole of the macrophage that the conditioned media is generated from. These experiments would be important to the field and provide illumination into how MMRd and MMRp ISCs are impacted by secretome from the immune cell compartment in the intestine.


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