Characterization of Colonic Macrophages in Mice Exposed to Social Stressor During Oral Challenge with *Citrobacter rodentium*.

Honors Research Thesis

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By

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Abstract: Chronic colonic inflammation, as well as exposure to psychological stressors, are well known risk factors for the development of colorectal cancer. Whether these two predisposing factors are linked, however, is not known. A murine social stressor, called social disruption (SDR), was used to test whether stressor exposure would increase colonic inflammation in mice infected with the colonic pathogen *Citrobacter rodentium*. When compared to non-stressed control mice infected with *C. rodentium*, stressor-exposure significantly increased pathogen-induced colonic histopathology, inflammatory cytokine gene expression, and the accumulation of F4/80+ macrophages in the colon. Other studies demonstrate that exposure to the SDR stressor without infection significantly increases the number of inflammatory monocytes in the spleen. Because splenic inflammatory monocytes can traffic to sites of inflammation, the goal of this study was to determine whether these accumulated colonic F4/80+ macrophages display an inflammatory phenotype (i.e., L6C<sup>hi</sup>CCR2<sup>+</sup>CD11b<sup>+</sup>) by using flow cytometry. To test whether colonic inflammation could be significantly enhanced by colonic macrophages, an adoptive transfer model was also used. In this experiment, monocytes isolated from the spleens of stressed mice were introduced into naïve mice, which were later infected with *C. rodentium*. It is expected that these non-stressed recipient mice will display similar inflammation and pathology as stressed mice. If proven true, it would mean that the stressor-primed splenic inflammatory monocytes are responsible for enhanced colonic inflammation. Increases in colonic macrophages may account for stressor-induced increases in colonic inflammation. Future studies will test the hypothesis that this increased inflammation predisposes animals to developing colorectal cancer.
1. Introduction

Psychological stress has been shown to have profound effects on physiological functions, especially in disease pathology (Bartolomucci et al., 2007). Importantly, among the different functions that are affected is the immune system. Stress can have a complex effect on the immune system, in some situations enhancing it, and others depressing it; however in most cases the effects are deleterious. Many of these negative effects are due to enhancement of the inflammatory immune response, which can lead to chronic and pathological inflammation. Inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn’s disease are particularly sensitive to stress, being linked to causing new flare-ups. Furthermore, chronic inflammation and stress have been implicated in increased risk for developing cancer, especially in the colon.

1.1 Psychological Stress:

Psychological stress can be defined as an organism’s physiological and behavioral response to a perceived potential danger. Stress alters and modulates many of the body’s normal functions in order to prepare the organism to cope with the potential danger, through a “fight or flight” response. When an organism encounters a stressor, two major pathways are activated which bring about these modulations (Bartolomucci, 2007). One of these pathways involves the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the release of glucocorticoids (Leonard et al., 2005). Glucocorticoids (cortisol in humans, corticosterone in mice) stimulate an increase in gluconeogenesis to ensure high glucose availability for ready use in quick bursts of energy (Khani et al., 2001). Additionally, glucocorticoids generally have immunosuppressive activity and are effective in reducing inflammation (Coutinho, 2011). The second major system
is the sympathetic nervous system (SNS), which innervates almost every organ in the body and is responsible for the well known “fight or flight” response (Bartolomucci, 2007). Activation of the SNS results in the almost immediate release of catecholamines such as epinephrine and norepinephrine (Carrasco et al., 2003). Catecholamines have many immunomodulatory behaviors, mainly regulatory and suppressive roles, such as decreasing TNF-α production by macrophages (Nance et al., 2007).

1.2 Stress Effect on the Immune System:

There is a widely acknowledged intimate relationship between stress and the immune system (Bartolomucci, 2007), (Pruett et al., 2003), (Segerstrom et al., 2004). The relationship is bi-directional: stress is capable of modulating the immune system, and the immune system is capable of inducing a stress response. Generally, stress is known to suppress the immune system. The release of glucocorticoids has strong immunosuppressive and anti-inflammatory effects, and they are known to inhibit the activity of lymphocytes and macrophages (Da Silva et al., 1999), (Reiche, 2004). However in many cases, stress has been shown to enhance the immune response, especially in inflammatory and autoimmune diseases (Dhabhar et al., 2002), in part by causing a redistribution of immune cells throughout tissues and the blood (Dhabhar et al., 2012).

Social stress in particular is a well defined and effective laboratory stressor (Bartolomucci, 2007). Venzala et al. examined the comparability of murine social stressors as models for human psychological disorders. They found that their stressor models significantly increased anxiety-like and other abnormal behavior over a range of behavior tests. Although they concluded social stressors are not perfect models for clinical depression, they acknowledged that
they accurately model many of the symptoms and behaviors of depression and anxiety-related disorders (Venzala et al., 2012).

Social disruption (SDR) is a well defined social stressor in which subordinate young mice are attacked and defeated by an older aggressive mouse over a span of 2 hours for 6 consecutive days. Defeated mice have been shown to have elevated levels of systemic corticosterone as well as modulated behavior (Avitsur et al., 2001). The SNS is also activated during SDR, as are brain regions associated with the activation of the SNS. Importantly, it has been reported that SDR can increase CD11b+ myeloid-derived leukocytes, such as neutrophils and monocytes both in circulation and in the spleen (Engler et al., 2004). Furthermore, splenocytes isolated from these stressed mice have been shown to have a decreased sensitivity to glucocorticoid inhibition in addition to expressing higher levels of inflammatory cytokines upon antigen stimulation (Bailey et al., 2003).

1.3 Inflammation

Inflammation is marked by the release of cytokines that stimulate the symptoms that are associated with the inflammatory response. Two well known markers are TNF-α and IL-1. TNF-α is important in basic immunity against pathogens including bacterial, viral and parasitic infections. TNF-α is produced primarily by activated macrophages and T cells. It precipitates the activation of complex and numerous pathways that culminate in the activation of NF-κB, a transcription factor pathway which has been shown to be pivotal in 221 other molecular associations (Bouwmeester et al., 2004). TNF-α stimulates the production of different selectins on vascular endothelial cells as well as vasodilatation, both of which are essential for recruiting leukocytes out of circulation and into the tissue. TNF-inhibiting drugs effectively treat a wide
range of chronic inflammatory diseases (Vincent et al., 2012) including Crohn’s disease (Hanauer et al., 2002). TNF antibodies can also prevent septic shock when injected with a lethal dose of bacteria (Tracey et al., 1987).

There are two forms of interleukin-1, IL-1α and IL-1β, that play important roles in the inflammatory response and inducing the expression of many other inflammatory genes (Dinarello et al., 2009). Exposure to the SDR stressor has been shown to lead to increases in both TNF-α and IL-1 upon stimulation (Engler et al., 2004).

Chemokines are a subclass of cytokines known for their role in leukocyte chemotaxis and homing. Chemokines are produced by cells at the site of infection, and incoming leukocytes travel up the concentration gradient towards the site. They can also be used to concentrate immune cells in specific locations, such as lymphoid tissue (Bachmann et al., 2006). CCL2 is an important inflammatory cytokine. It can be secreted by a range of cells including monocytes, macrophages, T cells, fibroblasts, endothelial cells, and mast cells. The cell receptor for CCL2 is CCR2, and is commonly found on proinflammatory cells such as infiltrating monocytes (Shachar et al., 2013). CCR2 is essential for monocyte recruitment and K/O mice exhibit significantly reduced numbers of leukocytes in tissue (Kuziel et al., 1997).

Inflammation in the colon is of particular interest because of its association with inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn’s disease, and its predisposal for developing colorectal cancer (CRC). Furthermore, colonic inflammation has strong correlations with stress.

The lumen of the colon is home to approximately ten times more bacterial cells than there are human cells in the body. These bacteria are commonly referred to as the colonic microbiota and have been shown to have numerous beneficial effects for the host. The epithelium of the
colon provides a physical barrier to contain the bacteria both through tight junctions and the production of a mucosal layer. The lamina propria, which lies directly beneath the epithelial layer, contains a wide variety of immune cells including macrophages, neutrophils, dendritic cells, natural killer T cells, and T and B cells. There is an intricate balance between these immune cells and the microbiota. The immune cells must be prevented from activating against all the normal microbiota, but also must be able to recognize and attack pathogens. Dysfunction of this balance can lead to chronic inflammation in the colon, which can lead to pain, discomfort, tissue pathology, and potentially the removal of the colon or development of CRC (Abraham et al., 2009). One type of cell that is involved with chronic inflammation in the colon is the macrophage.

1.4 Macrophages

Macrophages are generally tissue associated leukocytes of the innate immune response. They are mononuclear phagocytes, and play a vital role in orchestrating the initial immune and inflammatory responses. Monocytes are myeloid-derived cells in the blood stream, which differentiate into macrophages upon leaving the blood and entering the tissue.

Both monocytes and macrophages are highly heterogenous, and display a wide range of phenotypes based on their tissue location and function (Gordon and Taylor, 2005). Different macrophage phenotypes can have diverse and contradictory behaviors including phagocytic, inflammation-promoting, inflammation suppressing, cancer-promoting, cancer-suppressing or wound healing. F4/80 is a cell surface marker that is especially prevalent and highly expressed on macrophages in tissue, but lower levels can be found on dendritic cells and activated eosinphils. It is commonly used as a marker for macrophages in the lamina propria, where it is
highly expressed (Gordon et al., 2011). Important for the colon are inflammatory and anti-inflammatory macrophages, often roughly referred to as M1 and M2, respectively. The M2 phenotype is associated with wound healing, tissue restructuring and anti-inflammatory behavior. Arginase, an enzyme which competitively inhibits iNOS activity, and TGF-β production are associated with M2 phenotypes (Martinez et al., 2008). The M1 phenotype is associated with inflammation and marked by TNF-α, iNOS and CCL2 production. TNF-α plays a crucial role in recruiting monocytes/macrophages to sites of infection, inflammation and tissue damage, as well as organizing cellular structure (Locksley et al., 2001). Although TNF-α can have anti-tumor properties, over-expression can lead to cancer through a number of pathways, including growth and angiogenic factors, cellular restructuring, and most importantly for this study, through inflammation, recruitment of macrophages and stimulation of iNOS (Szlosarek et al., 2001). The enzyme iNOS is expressed by inflammatory macrophages and leads to the synthesis of nitric oxide (NO). NO and its byproducts can undergo a number of disruptive reactions with DNA, many leading to cell death, but some leading to genetic mutations (Ambs et al., 1998). Tumors are associated with high levels of iNOS, and inhibition of iNOS has been shown to significantly decrease adenocarcinoma development in the pancreas of hamsters (Takahashi et al., 2008), as well as in an azoxymethane-induced model for CRC in rats (Takahashi et al., 2006).

1.5 Citrobacter rodentium

*Citrobacter rodentium* is a noninvasive murine pathogen which colonizes the epithelium of the colon and induces disease pathology similar to enteropathogenic *Escherichia coli* (EPEC) in humans. Previously classified as *Citrobacter freundii* biotype 4280, *C. rodentium* strains were shown to contain genes identical to eaeA and eaeB in *E. coli*, which are necessary for the
characteristic attaching and effacing lesions associated with EPEC infections (Schauer et al., 1995). Oral infections with *C. rodentium* result in a well defined disease known as transmissible colonic hyperplasia which is characterized by thickening of the mucosal layer, epithelial cell proliferation, goblet cell depletion and elongation of intestinal crypts (Bhinder et al., 2013), (Higgins et al., 1999).

Importantly, infections with *C. rodentium* have been shown to be effective models for inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn’s disease (Higgins et al., 1999). Higgins *et al.* demonstrated that the immune response to *C. rodentium* was marked by high percentages of CD4$^+$ T-cells, increased IL-12, and TNF-α; all of which indicate a Th1 response. They also noted that the infiltration patterns of the T-cells and macrophages bore strong resemblance to the patterns seen in the chronic inflammation of IBDs.

### 1.6 Cancer

The intricate relationship between stress, the immune system and inflammation have important implications for cancer growth and development. Understanding these relationships is vital for forming an effective cancer prevention and treatment regimen.

Psychological stress and other behavioral factors have been shown to play an important role in cancer development and progression (Lutgendorf and Sood, 2011), (Costanzo *et al.*, 2011). Stress has been linked to shorter survival in breast cancer patients (Giese-Davis and Spiegel, 2003). Exposing laboratory mice to experimental psychological stressors has been used to model these effects in humans, and in a murine breast cancer model, exposure to stress increased cancer metastasis by 30-fold. This effect was found to be due to stress induced activation of the sympathetic nervous system (Sloan *et al.*, 2010). These effects, however, are not
limited to breast cancer. Stressed mice have also been shown to develop skin cancer significantly quicker than non-stress mice in an ultraviolet light-induced squamous cell carcinoma model (Saul et al., 2005). The mechanisms by which exposure to psychological stressors can enhance cancer progression are not well defined, but could involve stressor-induced increases in inflammatory responses.

Several studies have demonstrated a key relationship between chronic inflammation and cancer growth and development (Colotta et al., 2009). This effect is likely bidirectional. Inflammatory genes have been shown to be induced by oncogenes. For example, in an in vitro thyroid cancer cell model, the RET/PTC1 oncogene induces the expression of inflammatory genes, such as the gene encoding for CCL2 (Borrello et al., 2005). CCL2 recruits inflammatory monocytes, as well as activated T cells, to sites of inflammation where they produce inflammatory cytokines, like TNF-α and IL-1β, as well as reactive oxygen and nitrogen intermediates (Daly et al., 2003). These inflammatory cytokines and reactive intermediates have been shown to promote tumorigenesis through induction of a pathway that inhibits DNA mismatch repair enzymes and increases angiogenic factors (Jung et al., 2004) (Koshiji et al., 2005). While the cancer-inflammation relationship is important for all cancer types, it is especially apparent in colorectal cancer. Diseases marked by chronic colonic inflammation, such as inflammatory bowel diseases (IBD), have been shown to greatly increase the likelihood of developing colorectal cancer (CRC) (Jawad et al., 2011). It is estimated that only 20% of CRC cases are associated with hereditary causes (Rustgi, 2007), while the remaining portions are induced by environmental factors, and overwhelmingly those which induce inflammation and colitis (Terzic, et al., 2010). A collation of data from 19 articles suggests that 30 years after diagnosis with IBD, an average of 1 in 5 cases will result in CRC diagnosis, and about 50% of
those cases result in death (Lakatos et al., 2008). Importantly, the general risk of developing CRC can be significantly reduced through regular use of anti-inflammatory drugs such as aspirin (Chan et al., 2007). Thus, it is evident that colonic inflammation significantly increases CRC progression.

M1 macrophages create an inflammatory environment ideal for tumor formation as previously described, while M2 is the phenotype expressed by tumor-associated macrophages (TAM) (42). Tumors have been shown to produce CCL2, a chemotactic cytokine for CCR2+ inflammatory macrophages (Sica et al., 2008). Thus, stressor-induced increases in colonic macrophages, particularly during chronic colonic inflammation, could result in an internal environment conducive to the formation of colorectal cancer.

**Purpose:** The purpose of this thesis was to characterize macrophages in the colon during stressor-enhanced pathogen-induced inflammation, and to test the hypothesis that cytokines associated with M1 macrophages will be increased in stressor-exposed mice. Another goal of this thesis examine if social stress and colonic inflammation increases susceptibility to developing CRC.

### 3. Methods and Materials

#### 3.1 Mice and Social Disruption

Wild type, male C57BL/6 mice, aged 6-8 weeks, were purchased from Charles River Laboratories (Wilmington, MA) and housed 3 per cage in an AAALAC-approved facility. Mice were kept on a 12 hr light:dark schedule with lights on at 0600 hrs and given food and water *ad libitum*. After arrival, mice were given one week for acclimation, and observed for any sign of
inter-cage aggression. Social disruption involves introducing an aggressive male mouse into the cage of the SDR mice for two hours per evening, from 1700 to 1900 hrs, for six consecutive evenings. During this time, the aggressive male attacks and repeatedly defeats the young mice. SDR cages were observed for 20 min at the beginning of each cycle to ensure adequate fighting. Control mice were left undisturbed, and monitored for inter-cage fighting. After the first cycle of SDR, all mice were infected via oral gavages with 100 μl of *Citrobacter rodentium* strain DBS120 (pCRP1::Tn5) in PBS at a concentration of about 1-3 x 10⁷ CFU/ml. Mice were sacrificed on days 0, 6, 12, and 24 post-infection by CO₂ inhalation. Colons and spleens were removed and weighed, and snap-frozen in liquid nitrogen.

On days 1, 3, 6, 12, and 24 post-infection, stool was collected and cultured to determine extent of *C. rodentium* infection. Stool was homogenized and serially diluted in PBS. The *C. rodentium* used had a kanamycin resistance cassette, and therefore aliquots of the homogenized stool were plated on Lactose MacConkey agar (Becton Dickinson) supplemented with kanamycin (40 μg/ml) to select for the pathogen. Plates were incubated at 37 °C overnight without CO₂, and the number of colony forming units (CFU) per gram of fecal matter determined.

3.2 *Measurement of gene expression by Semi-Quantitative Real Time PCR Analysis*

On days 0, 6, 12, and 24 post-infection, colons were harvested and total RNA was extracted using Trizol reagent as per manufacturer’s protocol (Invitrogen, Carlsbad, CA). After isolation, total RNA was spectrophotometrically quantified so that a reverse transcription reaction of 2 μg of RNA could be performed to synthesize cDNA. The reverse transcription reaction makes a DNA copy (cDNA) of an RNA molecule, using the Avian Myeloblastosis Virus (AMV) Reverse Transcriptase enzyme (Promega Corporation, Madison, WI). These
samples then underwent Real-time Polymerase Chain Reaction (PCR), in order to amplify the nucleotide sequences of interest, which included mRNA for TNF-α, iNOS, CCL2, arginase, and TGF-β. The housekeeping gene that was used was 18S. Primers and probes were synthesized by Applied Biosystems.

The PCR mixture consisted of 2.5 μl of cDNA, 2.5 μl of primer mix, 2.5 μl of probe (or SYBR Green for arginase and TGF-β), 5 μl of sterile H₂O, and 12.5 μl of Tagman Universal Master Mix (PE Applied Biosystems, Foster City, CA) for a final volume of 25 μl. After an initial 2-min cycle at 50ºC followed by 10 min at 95ºC, the reaction ran for 40 total cycles, which consisted of a 15-s denaturing phase (90ºC) and a 1 min annealing/extension phase at 60ºC. The change in fluorescence was measured using an Applied Biosystems 7000 Prism Sequence Detector (PE Applied Biosystems) and analyzed using Sequence Detector version 1.0. The relative amount of transcript was determined using the comparative cycle threshold (Ct) method as described by the manufacturer.

3.3 Quantification of F4/80+ cells in colon tissue

Colons were harvested from SDR and HCC mice on days 0, 3, 6, 12, and 24 post infection and fixed in 10% formalin. Samples were then sent to the Vet Core where they were embedded in paraffin and sliced. The tissue was counterstained and treated with F4/80 primary antibody, and then a secondary antibody conjugated with horse radish peroxidase for visualization. The samples were photographed under a microscope at 100X. Every 5th photograph was quantified using Photoshop, as described previously (Eubank et al., 2009). In Photoshop, a positive pixel coloring was identified. The histogram was then run which turned all positive pixels black, and the rest of the pixels white. The percentage of pixels that were black was then determined. Pictures were cropped so that percentages reflected only colon tissue.
3.4 Lamina Proprial Lymphocyte Isolation and Flow Cytometry

Colons were harvested from SDR and HCC mice on day 12 post infection and flushed with HBSS. Excess fat tissue was removed from the colon, bisected, and then cut into 0.5 cm pieces which were placed in 1 mM dithioerythritol in HBSS solution. Samples were then shaken at 37 °C for 30 min, the supernatant removed, the tissue resuspended in 5% FBS in RPMI and collagenase (4.975 mg/25ml) solution, and shaken at 37°C for an additional 90 min. Supernatant was then filtered with a 70 μl cell strainer, washed, counted, and resuspended for flow staining. Fifty thousand cells were stained with 1 μl of FITC labeled CD45, PerCP labeled F4/80, and APC labeled CD11b in panel one and CD45-FITC, CCR2-PE, and CD11b-APC in panel two. Two panels for lymphocytes were also used, with antibodies for CD45-FITC, CD3-PE, CD4-PerCP and CD8-APC. CD45 is a marker used to identify leukocytes in general. CD3 is a marker used to identify T cells, CD4 and CD8 are markers for helper and cytotoxic T cells. Expression of these cell markers was determined on a Becton-Dickinson FACSCaliber four color cytometer, and 10,000 events were recorded. Flow results were gated first for mononuclear cells via forward and side scatter, then CD45+ cells, then CD11b+ cells, then CCR2+CD11b+ cells. A gate was set on CD45+ cells to determine the percentage of mononuclear cells that were CD45+. Within the CD45+ cells, a gate was set to determine the percentage of CD45+ cells that were CD11b+, and what percentage of cells were both CD11b+ and CCR2+.

3.5 AOM Study

In a second study, mice subjected to SDR in combination with azoxymethane (AOM) to assess colonic tumor formation. One week after arrival, the mice were given an inter-peritoneal
(IP) injection of 100 μl of either AOM at a concentration 1 mg AOM per 1 kg of body weight, or PBS. The first cycle of SDR was started one week after IP injections and C. rodentium was gavaged afterwards. Five more cycles of SDR were performed, and then the mice were left undisturbed until 20 weeks post-IP injection. Body masses were measured every other week, starting on week 8. At week 20, mice were euthanized by CO₂ inhalation. Livers, spleens, and colons were removed and weighed, colon length was measured, and tissues were snap-frozen in liquid nitrogen. Before freezing, colons were examined for signs of tumors, contents were removed, and the tissue was bisected longitudinally into two equal pieces. Half was frozen for storage, and half was fixed in 10% formalin for hematoxylin and eosin (H&E) staining, and histopathology analysis.

3.6 Statistical Analysis

To determine the differences between SDR stressor mice and HCC control mice, two-factor analysis of variance (ANOVA) with group (i.e., HCC vs. SDR) and day (i.e., 0, 6, 12, and 24) as between subject variables. For studies involving AOM, a third factor was added (i.e. AOM vs. PBS). The level of statistical analysis was set at p<.05. SPSS for Windows version 19 (SPSS, Chicago, IL) was used for all analyses.

4. Results

4.1 Macrophage Characterization

The data indicates that SDR increased the number of C. rodentium could be colonized from the stool, with the infection peaking on day 12. The SDR group had a marginally significant increase in C. rodentium across the 24 day period (F(1,64)=3.577, p=.064), highest on day 12 post-challenge. Non-stressed HCC mice tended to show little or undetectable amounts of
C. rodentium (figure 1). The detectable range for this experiment was from log(10) 2.6 to log(10) 7.2 CFU/gram of fecal matter.

Figure 2 shows tissue samples after staining with F4/80 antibody. Positive cells shown on the left are visualized by a secondary antibody conjugated to horse radish peroxidase, which turns brown. Photoshop was used to convert positive brown cell pixels to black, and all other pixels to white. The percentage of black pixels in the area of the tissue was then determined using Photoshop. SDR significantly increased the percentage of F4/80+ pixels in colon tissue over HCC over the course of the infection (F(4,26)=3.12, p<.05). Post-hoc testing indicated this was due to a significant increase on day 12 post-infection.

Figure 3 shows the results for real time PCR performed on colon tissue from stressed and non-stressed mice. Figures 3A and 3B show genes associated with M2 phenotypes, namely arginase and TGF-β. Although the mean level of TGF-β mRNA was lower in mice exposed to SDR compared to control mice, this difference did not reach statistical significance (F(1,16)=0.16, not significant). Stressor exposure did not effect arginase mRNA levels (F(1,16)=0.59, not significant). However, as shown in graphs C, D, and E, expression levels of genes associated with M1 phenotypes appear to change with stress. TNF-α expression was found to be significantly increased in SDR over HCC across days (F(3,24)=3.172, p<.05) with significant differences occurring on day 6 post-infection. Expression of iNOS also significantly increases with stress across days (F(3,24)=3.529, p<.05) with SDR being higher than HCC on days 6, 12, and 24 post-challenge. However, CCL2 was not found to be significantly changed (F(3,24)=9.337, not significant).

Figure 4 shows the results of flow cytometry performed on the leukocytes isolated from the lamina propria of the colon. As seen in figures 4A and 4B, there are more CCR2+CD11b+
events in SDR mice than in HCC mice. Figure 4C shows that stress induces a marginally significant increase in CD11b+ cells ($t(7)=2.23$, $p=.06$) and a marginally significant increase in double positive CCR2+CD11b+ cells ($t(6)=2.16$, $p=.07$). There was not a significant change in either CD4+ cells ($t(4)=0.23$) or in CD8+ cells ($t(4)=0.95$).

4.2 Azoxymethane Study

Figure 5 shows the levels of *C. rodentium* that were cultured from stool. The groups which received SDR, PCS (non-AOM PBS injection, *C. rodentium* infection, SDR) and ACS (AOM injection, *C. rodentium* infection, SDR) had a significantly increased colonization over the non-stressed groups, ACH (AOM injection, *C. rodentium*, HCC) and PCH (non-AOM PBS injection, *C. rodentium* infection, HCC) ($F(3,64)=4.37$, $p<.05$) across days with highest colonization occurring on day 12. The PBS mice also had significantly higher colonization over the AOM mice ($F(1,64)=11.66$, $p<.001$)

Masses of spleen, liver, and colon were measured. There appeared to be no significant trend between any of the variables. The same seemed to be true for the length of the colon.

Table 2 shows histopathology scores assigned to colon tissue harvested from week 20 post-injection. Tissues were assessed by a blinded pathologist for the listed inflammation pathology and given a score between 0 and 4, where 0 is healthy tissue and 4 is the most severe. There were no signs of continued inflammation in any of the samples, or indicators of tumors or pre-tumors.

5. Discussion
A previous study demonstrated that mice exposed to the SDR stressor during oral challenge with *C. rodentium* shed higher levels of *C. rodentium* into the stool and an increase in *C. rodentium*-induced pathology (Galley *et al.*, In Preparation). One measure in the pathogen colitis index is the accumulation of leukocytes in the colonic tissue, however, previously it was not known which types of leukocytes accumulate in the colon. The current study used immunohistochemistry for F4/80, to demonstrate that F4/80\(^+\) cells increase with SDR and *C. rodentium* infection. Since F4/80 is generally a marker for macrophages, it is likely that it is F4/80\(^+\) macrophages that are increasing with stress. However, dendritic cells (DC) can also display an F4/80\(^+\) phenotype, so it is possible that cells in addition to macrophages increase in the colon during stressor exposure.

Working with the hypothesis that macrophages are increasing with stress, we sought to further characterize the macrophages. Since macrophages can be either pro-inflammatory M1 type or anti-inflammatory M2 type, we performed real time PCR on colon tissue from day 12 mice. We looked for gene expression associated with the two different phenotypes. Expression of TNF-\(\alpha\), and inducible nitric oxide synthase (iNOS), genes associated with M1 macrophages were increased, while arginase and TGF-\(\beta\) expression, genes associated with M2 macrophages, had no significant change. Although these cytokines can be made by other cell types they have been used as markers for characterization (Martinez, *et al.* 2008). Thus, it seems reasonable that the macrophage population increased in the colon is of the M1 phenotype.

To further characterize the colonic macrophage population, flow cytometry was performed on lymphocytes isolated from the lamina propria of the colon. It was shown that of CD45\(^+\) cells (a marker for leukocytes), stress increased both CD11b\(^+\) cells and double positive CCR2\(^+\)CD11b\(^+\) cells. Other studies have also demonstrated increased populations of
CCR2⁺CD11b⁺ cells present in the colon during inflammation, and have found these cells to be cytokine producers (Bain, 2012). Flow analysis also revealed that CD4⁺ and CD8⁺ cells remained unchanged between stressed and unstressed animals, suggesting that increased T-cell numbers are not associated with stressor-enhanced inflammation.

Even though inflammatory macrophages and monocytes can be protective against bacterial infection (Fujiwara and Kobayashi, 2005), they can also contribute to uncontrolled colonic inflammation (Platt et al., 2010). Platt et al. demonstrated that they could isolate two distinct macrophage populations in the gut, F4/80⁺TLR2⁺CCR2⁻CX3CR1hi macrophages, which appear to be M2 type, and F4/80⁺TLR2⁺CCR2⁺CX3CR1int macrophages, which appear to be M1 type. They isolated the M2 type macrophages from resting colons, and found that they produced little to no TNF-α upon LPS stimulation. They isolated M1 type macrophages from colons during experimental colitis, and found that they produced high levels of TNF-α upon stimulation. They also showed that CCR2 knockout mice showed reduced M1 type macrophages, and also reduced levels of colitis, suggesting that these macrophages are contributing to the inflammation. Conversely, Hunter et al. have shown that a tape worm *Hymenolepis diminuta*, which has the ability to alternately activate macrophages, can reduce colitis in a DSS model. These alternately activated macrophages have increased expression levels of arginase and appear to be of the M2 phenotype, although further characterization was not performed (Hunter et al., 2010).

Chronic inflammation is a well known predisposing factor for developing cancer (Coussens and Werb, 2002) (Perwez-Hussain and Harris, 2007), especially in the colon (Jawad et al., 2011), because the inflammation creates an environment ideal for carcinogenesis. Enzymes such as iNOS produce free radicals which can induce mutations in host DNA. Inflammation is also often marked by an increase in growth factors, nutrients, and blood supply, all of which are
vital for the proliferation of cancer cells. Furthermore, colonic inflammation is often marked by rapid epithelial cell proliferation, a process which if mutated to become constitutive, could easily lead to tumor formation.

Stressor exposure increases *C. rodentium* induced pathology at the peak of infection (Galley *et al.*, In Preparation). In this study, we did not find evidence that the increased inflammation increased susceptibility to development of CRC. In fact, tumors or pre-tumor histological activity was not found in any animal in this study. It is important to note however, that the chronicity of inflammation is an important factor for CRC development. In our model, we know that stressor-enhanced colonic inflammation peaks around day 12 post-pathogen, and that both infection and inflammation is essentially resolved by day 24 post-infection (Galley *et al.*, Unpublished Observation). Thus, it is possible that a more prolonged inflammatory response would be more likely to induce CRC development.

However, this study was based off of studies by Suzuki *et al.*, which used dextran sodium sulfate (DSS) as the mode for inducing colonic inflammation. Suzuki’s lab demonstrated that CRC could be induced with a single injection of AOM followed a week later by a week of DSS administration, whereas previous labs used multiple weekly injections of AOM (Suzuki *et al.*, 2003). Our study simply replaced the DSS step with *C. rodentium* infection and SDR. DSS induces a similar, albeit more severe, inflammatory response in the colon, and resolves itself in a similar time frame (Yan *et al.*, 2009). Since the inflammation induced by DSS lasts for a similar time frame to our lab’s model, it seems less likely that a short duration of inflammation was the responsible for a lack of tumor formation. It is also possible that Suzuki’s model is effective because DSS induces a more severe and disruptive colitis than our model.
Furthermore, our results show that mice that received a PBS IP injection had significantly higher *C. rodentium* colonizations over mice that received AOM. Thus, it is possible that AOM could have either interfered with *C. rodentium* colonization, or killed *C. rodentium* directly, leading to even less severe colitis.

Since CRC can be induced with AOM injections alone, it is possible that SDR still might increase susceptibility for CRC if more than one AOM injection was administered. It is also possible that a different form of stressor used by this lab, namely prolonged restraint, could also prove more successful, due to the more severe colitis it illicits.

**Conclusion:**

This study demonstrated that F4/80+ cells increase in the colon with social stress and pathogen challenge and that these macrophages are likely of the M1 phenotype. Gene expression levels that are associated with and are used to mark M1 macrophages are increased in stressed mice over non-stressed mice. Furthermore, flow analysis showed that these cells had markers commonly accepted as markers of inflammatory macrophages. Additional studies are needed to further confirm these results.
Figure Legends

**Figure 1: C. rodentium colonization during SDR.** Fecal sheddings were collected on days 1, 3, 6, 12, and 24 post-infection, homogenized and plated on Lactose Mackoney agar supplemented with kanamycin (40 μg/ml) at 37°C for 24 hrs. These data points represent the log(10) colony of forming units (CFU) per gram of fecal matter +/- standard error mean (SEM). The SDR group had a marginally significant increase in *C. rodentium* across the 24 day period (F(1,64)=3.577, p=.064), highest on day 12 post-challenge. n=6

**Figure 2: F4/80+ staining of colonic tissue during SDR.** Colon tissue was harvested from SDR and HCC mice on days 0, 3, 6, 12, and 24 post-challenge with *C. rodentium*, fixed in 10% formalin, embedded in paraffin. Figure 2A and 2C represent colon tissue stained with F4/80 primary antibodies and visualized with horse radish peroxidase secondary antibodies. F4/80+ cells are seen as brown. Samples were photographed at 100X and quantified using Photoshop. Positive pixels were turned black and negative white with a histogram in order to determine percentage of positive pixels, as shown in Figures 2B and 2C. Data points in Figure 2E represent mean F4/80+ pixel percentage +/- SEM. SDR significantly increased the percentage of F4/80+ pixels in colon tissue over HCC over the course of the infection (F(4,26)=3.12, p<.05). Post-hoc testing indicated this was due to a significant increase on day 12 post-infection. Day 12, n=6, day 0, 3, 6, 24 n=3

**Figure 3: Real Time PCR of mRNA isolated from colonic tissue during SDR.** Colon tissue was harvest from mice on days 0, 6, 12, and 24 post-challenge with *C. rodentium*, mRNA was isolated using Trizol, and cDNA was synthesized. Real time PCR was run using mRNA primers for TGF-β, ARG-1, TNF-α, iNOS, and CCL2. Data points on Figures 3A-3E represent mean n-fold increase of expression over control mice minus expression of housekeeping gene 18S +/-
SEM. Figure 3A shows the mean level of TGF-β mRNA was lower in mice exposed to SDR compared to control mice, but was not statistical significance (F(1,16)=0.16, not significant). Figure 3B shows stressor exposure did not effect arginase mRNA levels (F(1,16)=0.59, not significant). In Figure 3C shows TNF-α significantly increased in SDR over HCC group across days (F(3,24)=3.172, p<.05) with significant differences occurring on day 6 post-infection. Figure 2D shows expression of iNOS also significantly increases with stress across days (F(3,24)=3.529, p<.05) with SDR being higher than HCC on days 6, 12, and 24 post-challenge. Figure 2E shows CCL2 did not significantly change (F(3,24)=9.337, not significant). n=3

**Figure 4: Flow cytometry of day 12 colon tissue.** Colons from mice subjected to SDR and HCC were harvested; lamina proprial lymphocytes were isolated and stained with stained with CD45-FITC, F4/80-PerCP, and CD11b-APC in panel one and CD45-FITC, CCR2-PE, and CD11b-APC in panel two. Two panels for lymphocytes were also used, with antibodies for CD45-FITC, CD3-PE, CD4-PerCP and CD8-APC. Figures 4A and 4B show that there are more CCR2⁺CD11b⁺ events in SDR mice than in HCC mice. Figure 4C shows that SDR mice had a marginally significant increase in CD11b⁺ cells (t(7)=2.23, p=.06) and 4D shows a marginally significant increase in double positive CCR2⁺CD11b⁺ cells (t(6)=2.16, p=.07). Figures 4E and 4F show there was not a significant change in either CD4⁺ cells (t(4)=0.23) or in CD8⁺ cells (t(4)=0.95). n=3

**Figure 5: Figure 1: C. rodentium colonization during SDR and AOM.** Fecal sheddings were collected on days 1, 3, 6, 12, and 24 post-infection, homogenized and plated on Lactose Mackoney agar supplemented with kanamycin (40 μg/ml) at 37°C for 24 hrs. These data points represent the log(10) colony of forming units (CFU) per gram of fecal matter +/- SEM. The groups which received SDR, and ACS had a significantly increased colonization over the non-
stressed groups, ACH and PCH (F(3,64)=4.37, p<.05) across days with highest colonization occurring on day 12. The PBS mice also had significantly higher colonization over the AOM mice (F(1,64)=11.66, p<.001). n=6

**Figure 7: Organ masses and lengths at 20 weeks post-injection.** Mice were euthanized and their livers, spleens, and colons were removed and weighed. Colon length was measured. There were no statistically significant differences in group for spleen mass (F(1,40)=.446) not significant), liver mass (F(1,40)=3.895), colon mass (F(1,45)=0.04), or colon length (F(1,40)=1.622). n=6

**Table 1: Pathology Assessment of AOM Colon Tissue.** Colon tissue was harvest from mice 20 weeks post-injection, fixed in formalin, embedded in paraffin, stained with H&E, and scored by a pathologist based on a scale of 0 to 4, 0 being healthy. No changes were observed.
Figures and Tables

Figure 1

![Graph showing log(10) CFU/g vs. Day Post-infection for HCC and SDR lines.](image-url)
**Figure 2**

D:

- Percentage of F4/80+ pixels vs. Days post-infection for SDR and HCC groups.
Figure 3

A: TGF-β

B: ARG-1

C: TNF-α

D: iNOS

E: CCL2
Figure 4

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AOM group:

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Figure 5

![Graph showing logs of C. rodentium over days post-infection](image-url)
Figure 6

Liver

Spleen

Colon mass

Colon length

ACS  ACH  AVS  AVH  PCS  PCH  PVS  PVH

grams

ACS  ACH  AVS  AVH  PCS  PCH  PVS  PVH

grams

ACS  ACH  AVS  AVH  PCS  PCH  PVS  PVH

grams

ACS  ACH  AVS  AVH  PCS  PCH  PVS  PVH

centimeters
Table 1

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