A Titratable Necrotizing Enterocolitis Mouse Model

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ABSTRACT

Necrotizing enterocolitis (NEC) is an inflammatory gastrointestinal process that afflicts nearly 10% of premature babies born in the United States each year, with a mortality rate of 15 - 30%. NEC severity is graded using the Bell's classification system based on the presenting symptoms and radiological findings. Animal models are a valuable tool in studying the pathogenesis of this disease; however, prior models cannot control the severity of NEC induced, a critical aspect of the disease in human infants. Here, we aim to bridge this gap in knowledge through development of a murine model of NEC with graded characteristics.

Postnatal day 3 (P3) C57BL/6 mice were fed formula containing different concentrations (0.25%, 1%, 2%, and 3%) of dextran sodium sulfate (DSS), an osmotic agent previously shown to induce severe NEC in neonatal mice. P3 mice were fed every 3 hours over a 72-hour period. During feeding, we collected data on weight gain and behavior, which included activity, response, and body color. After feeding, at P6, we dissected the mice to check for bowel features and collect organs for immunohistochemistry and cytokine analysis. Mice fed formula only or 0% DSS were used as a control instead of breastfed mice. Since staying with the dam during the first week of life is vital to normal development in mice, using formula-fed mice as the control accounts for the handling bias that exists during feeding.

Comparing breastfed and formula-fed mice alone, weight gain was significantly greater in breastfed mice, and liver cytokine concentrations tended to be higher. In the established graded NEC model, clinical differences were seen among the mice fed different DSS concentrations. Over the course of NEC induction, mice fed higher concentrations of DSS lost weight quicker and exhibited greater clinical severity scores. External bowel scores (dilation, color, and friability) were considerably worse in mice fed higher DSS concentrations; although internal small bowel pathology showed intestinal disarray among mice fed all DSS concentrations. Staining for cellular proliferation marker Ki-67 in intestines shows that mice fed higher concentrations of DSS display less cell proliferation at the base of the small intestine crypts. Pro-inflammatory cytokine levels in the liver, to assess systemic inflammation downstream of the

gastrointestinal system, trended higher in mice fed with higher concentrations of DSS.

Here, we demonstrate that we have successfully characterized a graded newborn mouse model where NEC severity can be predictably induced. This mouse model will be a valuable tool in gaining greater insight and understanding of the pathophysiology of this devastating disease process and to understand its long0term complications, such as neurodevelopmental impairment.

INTRODUCTION

Necrotizing enterocolitis (NEC) is the most common intestinal emergency among preterm and term newborns. Nearly 10% of newborns admitted to neonatal intensive care units develop NEC during their hospitalization, of which the majority (90%) were born prematurely[1]. Despite numerous preclinical and clinical research studies in this field, NEC remains a leading cause of death and long-term neurodevelopmental disability among survivors[2, 3].

Patients with NEC exhibit a wide range of clinical, radiological and laboratory changes. The Bell's Classification system uses clinical and radiological findings to classify NEC severity into mild (I), moderate (II), and severe (III) categories[4, 5]. Patients with mild NEC can be managed with medical therapy such as bowel rest, antibiotics, and parenteral nutrition. Approximately 15 to 30% patients, however, will have their enterocolitis progress to bowel necrosis and perforation, thus requiring surgical intervention necrotic or perforated bowel[6]. The overall mortality for NEC ranges from 15 to 30%, and 50 to 70% NEC patients requiring surgery die[7, 8]. Of patients who survive an episode of NEC, nearly 60% of them will suffer from long-term neurodevelopmental and cognitive impairments, in addition to the intestinal and nutritional morbidity of enterocolitis[9].

The exact cascade and precipitating factors for NEC are an area of active research. Broadly, in NEC, bacterial translocation and the breakdown of the neonatal gastrointestinal epithelium results in colitis and subsequent activation of systemic inflammation, resulting in sepsis[10]. Work from animal models have demonstrated a role for toll-like receptor 4 (TLR4), the inflammatory cytokine cascade, and the microbiome in the pathophysiology of NEC[11-13]. How these processes interact and create the spectrum of symptoms in NEC are still unknown. Early, or stage I according to Bell's classification, NEC often presents with nonspecific and subtle changes that complicate the diagnosis[6]. With all current mouse models, NEC severity cannot be prospectively induced; enterocolitis severity in current models can only be categorized with postmortem examination of the intestines[14-16]. It would be useful to create a NEC model that can predictably recreate the severity of enterocolitis induced. This model would allow us to study early pathways involved with NEC and, furthermore, allow us to dissect and understand the complex physiological interactions and

checkpoints that result in sepsis and necrosis.

Building upon a NEC model described by Ginzel et al. (2017), our group has characterized a graded model of NEC[17]. By adding increasing concentrations of dextran sodium sulfate (DSS), an osmotic agent, to enteral gavage feedings of neonatal mice starting on the third day of life, we demonstrated a graded response in clinical characteristics and mortality, intestinal morphological changes, and hepatic cytokine and chemokine concentrations. This novel graded neonatal NEC mouse model will advance our understanding of the early stages of NEC, provide insight into the transition from early to more advanced NEC stages, and help to decipher the interplay between gastrointestinal, immune, and inflammatory systems.

MATERIALS AND METHODS

Animals

All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Stony Brook University, Stony Brook, NY, in concordance with the guidelines established by the NIH. Mice (C57BL/6, Charles River, Wilmington, MA) were bred and housed within the Stony Brook University's animal care and research facilities, which are part of the Division of Laboratory Animal Resources (DLAR).

Necrotizing Enterocolitis (NEC) Induction with Dextran Sodium Sulfate

Enterocolitis was induced in mice, as described by Ginzel et al. (2017)[17]. Mice were separated from their dams and placed in an incubator (36.8°C, Ohmeda Medical Ohio Care Plus Incubator) starting at 72 hours after birth. Animals were randomly assigned to control or experimental groups. Two hours after separation from the dam, pups were fed by orogastric gavage every three hours for 72 hours total. Pups were initially fed with 50 μL of Esbilac formula (PetAg, Hampshire, IL) supplemented with dextran sodium sulfate (DSS, Sigma-Aldrich, St Louis, MO). DSS supplementation in formula was 0.25, 1, 2, or 3% w/v (control was defined as formula alone or 0% DSS). Furthermore, formula-fed pups were compared to pups nursed by their dams (breastfed group). Gavage volumes were increased by 10 μL daily to account for weight gain (50 μL, 60 μL, and 70 μL for days 1, 2, and 3, respectively).

Animals were monitored every three hours for signs of NEC (rectal bleeding, lethargy, and abdominal distension). Body weight was recorded every 12 hours, beginning at the initiation of feeding (0 hours). The clinical sickness score (CSS) was assessed every 12 hours, as described in Zani et al. (2008)[15]. The first CSS was recorded 12 hours after the start of gavage feeds, and the last CSS was recorded 60 hours after the initiation of feeds. As per IACUC protocol, animals were euthanized if they exhibited apnea, rectal bleeding, lethargy, weight loss > 15% of total body weight per day, or no weight gain for two days. Tissues were prepared for analysis if animals were euthanized. All animals were euthanized after 72 hours. Organs were harvested for histological and immunological analysis.

Organ Histology

Gastrointestinal Preparation

Intestines were prepared, as described in Bialkowska et al. (2016)[18]. Briefly, after dissection, the intestines were placed in cold PBS. Mesentery and connective tissue were removed. External bowel score was assessed (consistency, color, dilatation)[15]. The bowel was flushed with modified Bouin's fixative (50% ethanol/5% acetic acid in dH_2O) and cut at the ileocecal junction. Using the Swiss-rolling technique, the bowel was fixed overnight in 10% formaldehyde at room temperature and transferred to cold 0.1M PBS the following day. The bowel was paraffin-embedded and sliced into 5 µm sections for hematoxylin and eosin (H&E) or immunohistochemical staining. The Research Histology Core Laboratory, Department of Pathology, Stony Brook University, performed tissue processing, paraffin embedding, slicing, and H&E staining.

Organ Tissue Preparation for Cytokine Measurements

Organ tissue samples, specifically livers, were collected into sterile microcentrifuge tubes, weighted, and subsequently homogenized in sterile endotoxin-free saline using sterile 2.3 mm zirconia/silica beads (Biospec Products Inc.; Bartlesville, OK), as previously described[19]. Samples were then centrifuged at 13,000 g for 10 min at 4°C, and supernatants were harvested and stored at -80°C for subsequent cytokine measurements.

Immunohistochemistry (IHC)

Intestinal IHC

5 µm intestinal paraffin slides were prepared, as described by Talmasov et al. (2015)[20]. Sections were deparaffinized in xylene, incubated in 2% hydrogen peroxide in methanol for 30 minutes, rehydrated in an ethanol gradient, then treated with 10 mM Na citrate buffer, pH 6.0, at 120°C for 10 min in a pressure cooker. The slides were blocked with 5% bovine serum albumin (BSA) in wash solution, 0.01% Tween 20, 1X Tris-buffered PBS (TTBS), and incubated with primary antibodies against Ki-67 and cleaved caspase-3 (Table 1) overnight at 4°C. Slides were washed with TTBS, incubated with a secondary antibody horseradish-peroxidase (HRP) probe from the MACH 3 Rabbit HRP Polymer Detection kit (Biocare

Medical, Pacheco, CA), washed with TTBS, and treated with a tertiary antibody red HRP polymer from the same kit. After TTBS washes, color was developed using the Betazoid DAB Chromogen kit (Biocare Medical, Pacheco, CA), counterstained with hematoxylin solution, Gill (Sigma-Aldrich, St Louis, MO), and stained blue in lithium carbonate. Slides were dehydrated using alcohol and xylene gradient before mounting.

Table 1. Primary (1°) antibodies used for IHC of intestine (paraffin) slices.

1° antibody	Description	Species	Dilution	Company	Catalog #
Intestine (paraffin)					
Ki-67	Cellular proliferation	Rabbit	1:200	Biocare Medical	CRM325B
CC3	Cleaved caspase-3	Rabbit	1:400	Cell Signaling	Ab#9661

Image Acquisition

Light Microscopy

Intestinal slides were acquired on an Olympus VS120 Virtual Slide Microscope (Olympus Corporation, Japan) at 20x magnification of stained paraffin slices. Images were processed using Olympus Viewer software, and representative regions were saved at 200x magnification (10x zoom of the original scan taken at 20x magnification). The regions selected contained areas of maximum necrosis in the small bowel and were imaged for cell count analysis.

Image Analysis

All experimenters were blinded to the treatment conditions during image analysis. Besides colitis evaluation, at least two independent individuals analyzed identical samples, and the results were compared to ensure consistency of analysis. The mean of cell counts across individuals was used as the cell count.

Intestinal Morphology Analysis

H&E-stained intestinal sections were scored based on Tanner et al. (2015)[21]. A minimum of three

animals were used for each condition for enterocolitis grading.

Intestinal Cell Count Methodology

Caspase-positive cells were counted in crypts within a 200x high power field image of intestinal slides. Three animals were analyzed for each condition. For Ki-67 counts, at least four and up to ten crypts were identified per 200x high power field image. Ki-67-positive cells in each crypt were counted. Intestines from at least three different animals per condition were analyzed.

Cytokine Analysis

Cytokine (IL-1α, IL-1β, IL-2, IL-6, IL-10, IL-12p40, IL-12p70, IL-17, CCL2, CCL3, CCL4, G-CSF, GM-CSF, IFN-γ, TNF-α) concentrations in blood plasma samples and tissue homogenate supernatants were determined with Bio-Plex Pro magnetic multiplex assays (Bio-Rad; Hercules, CA) and analyzed on the Bio-Plex 200 system with Bio-Plex Manager 5.0 software (Bio-Rad). Results were expressed as cytokine concentrations in pg per mL plasma and pg per mg protein concentration for supernatants of organ tissue homogenates. Duplicate technical replicates were used for all immunological studies. Protein concentrations in supernatant tissue homogenate samples were determined with the Bradford method (Bio-Rad Laboratories; Richmond, CA) and measured on a Spectramax 190 Plate Reader (Molecular Devices LLC; San Jose, CA).

Statistical Analysis

All statistical analysis was performed using GraphPad Prism software. In comparing cytokine and chemokine concentrations between breastfed and formula-fed groups, multiple unpaired t-tests were performed for each protein; a correction for multiple comparisons was performed using the Bonferroni-Dunn method and setting the p-value threshold at 0.05. One-way analysis of variance (ANOVA) tests were performed for bowel scores and Ki-67 counts, cytokines. Two-way ANOVA tests were performed for all weight data comparisons, behavior scores, and bowel pathology. All ANOVA tests were verified by a post-hoc Tukey analysis for comparison between all data pairs. A survival curve log-rank test was performed for mouse mortality during NEC feeding. All data is displayed as mean ± standard error of mean (SEM), and p-value < 0.05 was considered statistically significant.

RESULTS

Formula administration affects growth and cytokine expression in comparison to breastfed mice.

Mice fed formula alone gained weight at a lower rate when compared to those who remain with their dams and were breastfed. On average, formula fed mice gained less than 20% of their starting weight after the 3-day feeding protocol (Figure 1A). Breastfed mice, on the other hand, nearly doubled in weight between the ages of postnatal day 3 (P3) and P6. In general, prior literature has shown that separating neonatal mice from their mothers during this first week of life can drastically alter offspring development and behavior, presenting a concerning confounding variable in selecting a control for a NEC mouse model.[22, 23]

In addition to weight gain, mice fed breastmilk show differences in systemic cytokine and chemokine levels compared to formula-fed mice. Broadly, breastfed mice have higher concentrations of hepatic cytokines and chemokines (Figure 1B). Although these changes were not significant when adjusted for multiple comparisons, individual comparisons noted several cytokines and chemokines that were significantly different between the two groups. These cytokines and chemokines included IL-1β (p = 0.0236), TNF-α (p = 0.0263), IFN-γ (p = 0.0249), IL-10 (p = 0.0448), IL-12(p40) (p = 0.0104), IL-12(p70) (p = 0.0057), IL-17 (p = 0.0144), CCL2 (p = 0.0469), and CCL3 (p = 0.0475). Due to these differences between formula-fed and breastfed mice, the control used for comparison with this mouse model is the formula fed (0% DSS) mice.

Increased DSS supplementation leads to earlier abnormalities in clinical assessment status (higher clinical sickness scores) and diminished weight gain.

Over the course of the 72-hour protocol, a clinical sickness score (CSS) was used to assess neonatal mouse behavior[13]. Mice in the 2% DSS group began exhibiting clinical signs of illness (appearance, activity, and response to touch) as early as 12 hours of feeding, with increasing clinical sickness scores over time (Figure 2A and Supplemental Figure 1). In contrast, mice fed with lower concentrations of DSS (0%,

0.25%, or 1%) began exhibiting behavioral abnormalities at 36 hours. 2% DSS-fed mice demonstrated significantly higher clinical sickness scores when compared to 0%, 0.25%, and 1% DSS-fed mice at 36 hours, and also when compared to 0% and 0.25% DSS-fed mice at 60 hours ($p < 0.001$, two-way ANOVA with Tukey's post-hoc). Overall, the CSS worsened as feeds progressed, and supplementation with a higher concentration of DSS caused worse clinical sickness scores.

Approximately 50% of all the experimental mice survived the 72-hour feeding protocol. The other 50% of mice either died between feeds or needed to be euthanized, as required per IACUC protocol. Mice that died unexpectedly were omitted from the study. sMice in the 0%, 0.25%, and 1% DSS groups all had comparable Kaplan-Meier survival curves (Figure 2B) which differed significantly from those in the 2% and 3% DSS groups ($p < 0.001$, survival curve log-rank). All mice in the 2% DSS group died or were euthanized before 72 hours, and mice in the 3% DSS group died or were euthanized before 48 hours ($p < 0.0001$, survival curve log-rank, for 2% versus 3% DSS).

Weights were measured in 12-hour intervals throughout the entire feeding period to determine if different concentrations of DSS would differentially affect weight gain. DSS supplementation at higher concentrations resulted in initial weight gain followed by weight loss, whereas mice fed with DSS concentrations less than 2% consistently increased in their weight (Figure 2C); there was no statistically significant difference in overall weight gain among these low concentration DSS groups ($p = 0.3543$, two-way ANOVA with Tukey's post-hoc). The mean normalized weights of mice that survived to the final 72-hour timepoint were as follows (mean ± SEM): 0% DSS 1.13 ± 0.01, n = 20, 0.25% DSS 1.14 ± 0.02, n = 11, 1% DSS 1.11 ± 0.02, n = 13). The 2% DSS-fed mice lost weight between 36 and 48 hours, with a skewed increase in weight at 60 hours, (only 2 of 9 surviving mice could be measured at 60 hours of DSS feeding). Overall, mice who received high DSS concentrations demonstrated significant decreases in weight gain, clinical sickness scores, and survival rates compared to lower DSS concentrations and control mice. Since 2% and 3% DSS-fed groups both exhibited severe phenotypes with poor survivability and weight gain, the 3% DSS group was excluded from the remainder of this study.

DSS feeds induced histological small intestinal disarray and inflammation in neonatal mice.

Histologically, NEC initially causes enterocyte disorganization and recruitment of inflammatory cells to the intestines. This inflammatory reaction leads to villous sloughing that progresses to the intestines' muscularis portion, ultimately resulting in transmural necrosis and perforation[19]. Hematoxylin and eosin (H&E) staining of the intestines of DSS-treated mice are illustrated in Figure 3A. There is increasing derangement of villous organization, intestinal dilatation with patchy necrosis, and perforation seen on higher power (Figure 3A, top and middle rows). The external bowel score, as described by Zani et al. (2008), assesses gut color, dilation, and consistency or friability, which worsens in mice exposed to increasing concentrations of DSS (Figure 3B, Supplemental Figure 2)[13]. The external bowel scores are as follows (mean ± SEM): 0% DSS 0.6 ± 0.2, n = 18, 0.25% DSS 1.2 ± 0.2, n = 13, 1% DSS 4.0 ± 0.3, n = 18, 2% DSS 4.7 ± 0.4, n = 7). Mice fed 0%, or 0.25% DSS demonstrated similar bowel scores that were significantly lower than those of mice fed 1% or 2% DSS-fed mice ($p < 0.0001$, One-way ANOVA with Tukey's post-hoc). Intestines from 1% and 2% DSS mice had comparable bowel scores.

Consistent with the results described by Ginzel et al. (2017), enteral DSS administration induced primarily small intestinal injury in neonatal mice. These findings are in contrast to reports of DSS exposure in older mice, where the colon is primarily affected (Figure 3C)[15]. Mice that received even the lowest concentration of DSS (0.25%) demonstrate patchy villous disorganization. Intestines were scored according to the worst pathology observed in the slide. Intestines from mice fed 0.25% DSS and 1% DSS scored significantly worse compared to those from 0% DSS-fed mice ($p < 0.0001$ and $p = 0.0074$, respectively). Intestinal pathology in this mouse model localizes predominantly to the small intestine; thus, damage induced by DSS feeding in newborn mice mimics the pattern of NEC lesions found in patients.

DSS exposure suppressed intestinal cell proliferation without promoting apoptosis.

Tissue loss or injury can be mediated by increased cell death or decreased proliferation. Intestines were assessed for the presence of cellular apoptosis and/or proliferation by immunohistochemical staining

for cleaved caspase-3 (CC3) or Ki-67, respectively. For all experimental groups, small intestinal crypts did not show positive staining for CC3, indicating that apoptosis is not a feature of DSS-induced small intestinal injury (data not shown). In contrast, Ki-67 positive (Ki-67+) staining of small intestinal crypts of 2% DSS-fed mice was significantly suppressed compared to 0% or 0.25% DSS-fed pups (Figure 3A, bottom row, and 3D). The Ki-67+ cell counts per crypt were as follows (mean ± SEM): 0% DSS 13.2 ± 0.9, number of crypts – 27, 0.25% DSS 14.7 ± 0.8, n = 42, 1% DSS 13.2 ± 1.0, n = 16, 2% DSS 10.9 ± 1.1, n = 7. ($p < 0.0001$, one-way ANOVA with Tukey's post-hoc).

Pro-inflammatory cytokines and chemokines are found at higher concentrations in the livers of mice fed higher DSS concentration.

Cytokines and chemokines are small proteins circulating in the blood to modulate inflammation and facilitate communication between immune cells. As a result of the intestinal inflammation in NEC, changes in cytokine levels would be expected to correlate with the amount of DSS fed to the mice. Blood from the intestines drains directly to the liver via the portal venous system, making the liver a targetable organ for assessing cytokine levels. Several pro-inflammatory cytokines and chemokines (e.g., TNF-α, IL-6, IL-1α, IFN-γ) demonstrated significantly higher hepatic tissue concentrations among 2% DSS fed mice compared to the other experimental groups (Figures 3A-3E). In contrast, the hepatic concentrations of the anti-inflammatory cytokine IL-10 showed only a modest increase in hepatic tissue of 2% DSS versus 1% DSS fed mice, indicating a pro-inflammatory milieu (Figure 3F). The findings of the other tested cytokines and chemokines are shown in Supplemental Figure 3. IL-1β ($p = 0.1669$), IL-12p40 ($p = 0.1476$), and IL-17 ($p = 0.0798$), CC2L, CC3L, and CCL4 (Supplemental Figures 3A, 3B, 3D). Hepatic GM-CSF and CCL3 concentrations in 2% DSS mice were significantly elevated compared to all other experimental groups, IL-12p70, G-CSF, and CCL2 were significantly greater compared to r0% and 1% DSS, and CCL4 was significantly higher compared to 1% DSS fed mice. Overall, this pattern of hepatic cytokines and chemokines demonstrated a pattern of predominant pro-inflammation as opposed to decreased anti-inflammation.

DISCUSSION

Necrotizing enterocolitis continues to be a major cause of acute long-term morbidity in premature infants[6]. Novel and refined animal models are needed for to study the pathophysiology and etiology for this complex and multifactorial disease process[24, 25]. The clinical manifestation of NEC exists on spectrum of presentations that can begin with mild enterocolitis and progress to fulminant sepsis, intestinal necrosis, and, ultimately, death. We have characterized a newborn mouse model that predictably creates a graded clinical presentation of NEC. By modifying the concentration of DSS, we were able to recreate various severities of NEC as shown in our behavioral and clinical data, gross pathology analysis, and cytokine measurements[15].

Neonatal mice react differently to enteral DSS administration when compared to adult mice as demonstrated in Ginzel et al. (2017)[17]. Adult mice develop colonic colitis with DSS administration, while neonatal mice also develop small intestinal enterocolitis. Taking advantage of the DSS mouse model, we modulated the concentration of DSS – 0.25%, 1%, 2% and 3% DSS – to create a graded model of NEC.

Formula fed mice (0% DSS) demonstrate differential weight gain and cytokine levels when compared to mice who remain with their dams. Multiple studies have demonstrated that the stress of maternal separation, lack of breast milk, and lack of grooming by the dams can result in elevated stress hormones and changes in behavior[22]. Breast milk contains growth factors, antibodies and other biological byproducts that are not found in commercially available formula, these factors alone can explain the different pattern in growth and cytokine level observed in dam fed mice[26]. Interestingly, there was no difference in colitis score in breastfed mice and formula-fed mice suggesting that factors extrinsic to bowel development could contribute to the differences between breastfed and formula-fed mice. From our data, the handling of pups with enteral formula can induce cytokine and growth differences from breastfed mice; therefore, formula-fed (0% DSS) pups were used as the control in this paper.

Applying the clinical sickness score developed by Zani et al. (2019), mice treated with increasing concentrations of DSS developed worsening clinical sickness scores at earlier time points of the

experimental protocol than those exposed to lower concentrations[15]. Furthermore, our weight data corroborates these behavior measures. Mice fed higher concentrations of DSS also developed bloody stools sooner, thus requiring euthanasia, and demonstrated increased mortality when compared to controls (0% DSS) and mice fed lower concentrations of DSS.

Our gross pathological and microscopic findings confirmed that enteral DSS administration primarily induced small intestinal enterocolitis in neonatal mice with minimal colonic inflammation. Although there were no detectable differences in apoptosis as indicated by cleaved caspase-3 staining in our intestinal sections, increased DSS administration significantly diminished intestinal mucosal replication as demonstrated with Ki-67 staining. Clinically, higher concentrations of DSS administration resulted in more frequent observations of bowel distension, bloody stool, and friability[15]. We used a strict IACUC euthanasia protocol once animals were moribund or demonstrated gross bloody stool and weight loss, which may have accounted for the absence of caspase staining, especially in the 2% DSS group.

Our measurements of organ tissue inflammatory mediators demonstrated that enteral feeding of increasing DSS concentrations induced rising hepatic pro-inflammatory cytokine and chemokine concentrations. Therefore, worsening degrees of intestinal inflammation in our enterocolitis model led to increasing cytokine and chemokine expression in the liver, which is the first major organ downstream of intestines. Other NEC animal models have demonstrated increases in cytokine levels of IL-6, IL-8, TNF-α, and IFN-γ as well[11, 27]. With our model, we are able to distinguish cytokine levels between mild, moderate and severe NEC.

In conclusion, our established murine NEC model enables researchers to study the different severity stages of NEC. This allows detailed manipulation of the disease process to identify the pathways involved in the initiation of the physiological cascade associated with mild NEC. With this model, we will have better control in understanding the mechanisms that precipitate the systemic inflammatory response observed in patients. Long-term, this model will allow researchers to identify early targets for intervention and prevention of the neurocognitive consequences of NEC in premature babies.

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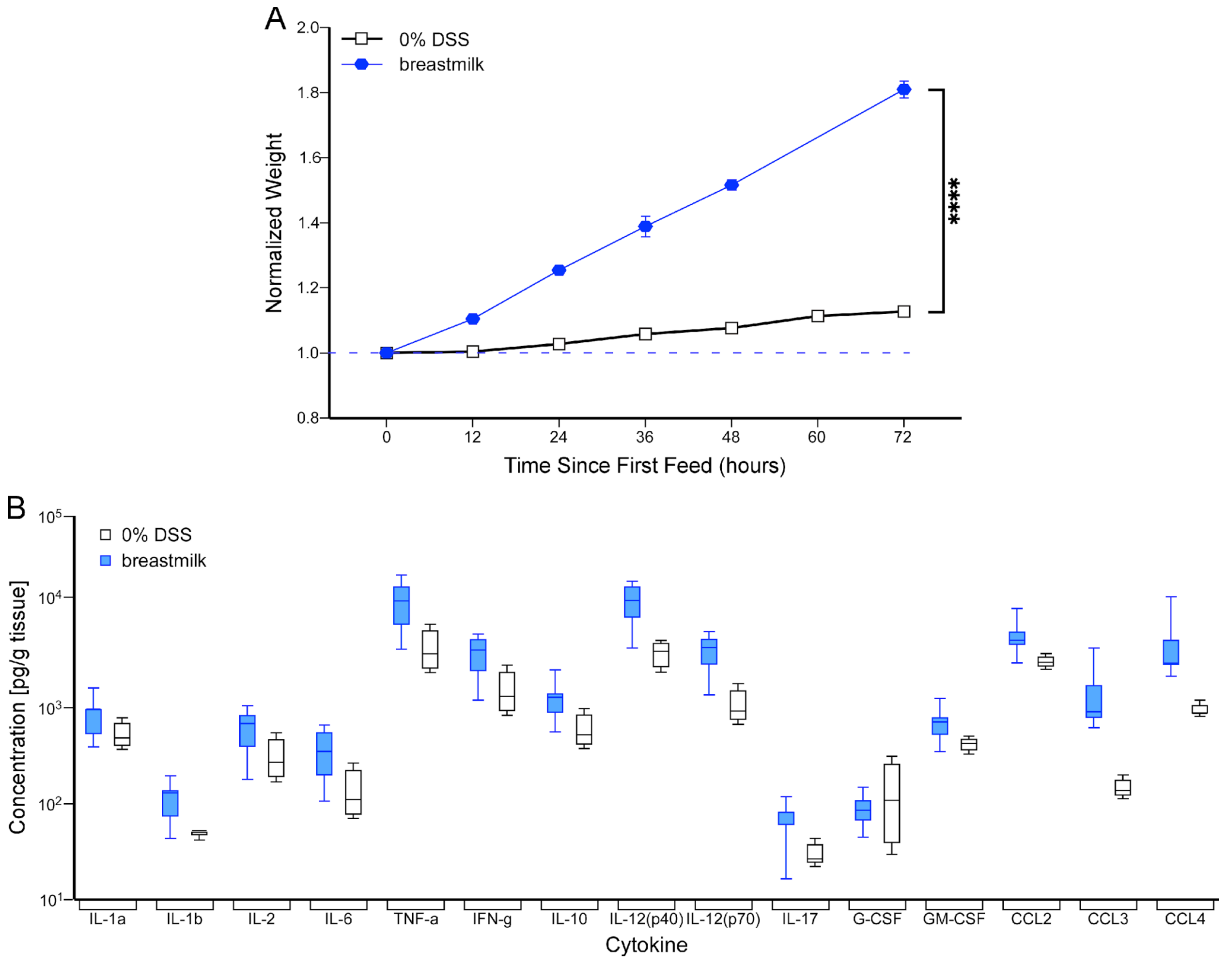


Figure 1. Breastfed mice exhibit notable differences in weight gain and cytokine profile compared to mice fed formula only (0% DSS).

(A) Breastfed mice gain weight more rapidly compared to formula-fed mice, $p < 0.0001$. Breastfed mice nearly double their weight within 72 hours. Two-way ANOVA with Tukey's post-hoc. Error bars are SEM. ****$p < 0.0001$. Number of mice for breastfed: n = 7, 0% DSS: n = 40.

(B) Hepatic cytokines trend higher in breastfed mice compared to formula-fed mice. Certain cytokines – IL-1b, TNF-alpha, IFN-gamma, IL-10, IL-12(p40), IL-12(p70), IL-17, CCL2, and CCL3 – are significantly different when analyzed independently but not upon adjustment for multiple comparisons. Multiple unpair t-tests with Bonferroni-Dunn method to adjust for multiple comparisons. Number of livers for breastfed: n = 9, 0% DSS: n = 4.

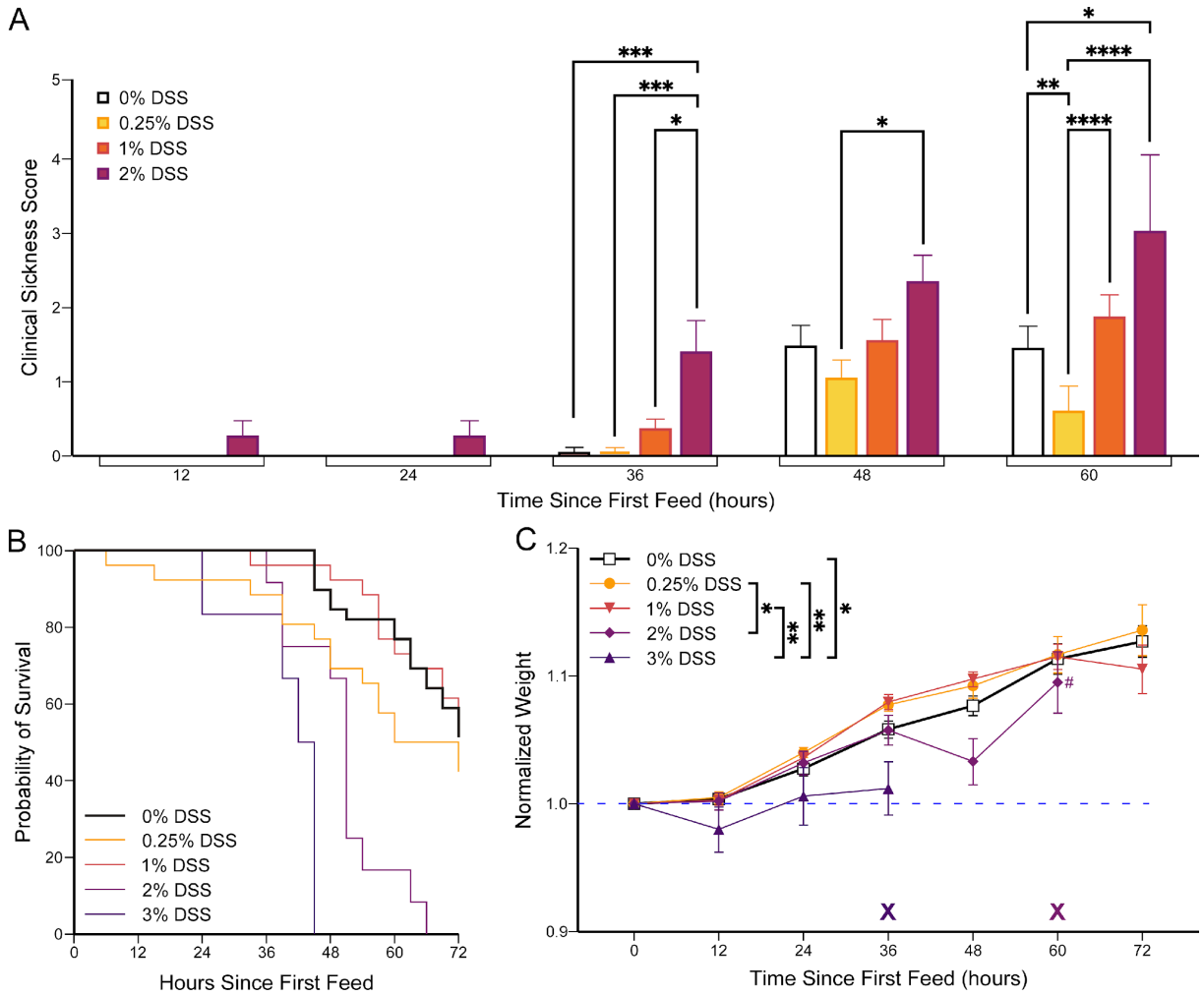


Figure 2. Dextran Sodium Sulfate (DSS) concentration correlates with severity of clinical sickness score (CSS), survival, and weight changes.

(A) CSS assessment of mice exposed to increasing concentrations of DSS. CSS increases more rapidly in animals exposed to higher concentrations of DSS. Two-way ANOVA with Tukey's post-hoc, $p < 0.0001$. Number of mice for 0% DSS: n = 29, 0.25% DSS: n = 26, 1% DSS: n = 23, 2% DSS: n = 7.

(B) Kaplan-Meier survival curve for mice throughout feeding protocol. Mice fed high concentrations of DSS (2% & 3%) survived for a shorter time versus lower concentrations. Survival curve, $p < 0.0001$.

(C) Different concentrations of DSS supplementation change weight gain pattern in neonatal mice. Two-way ANOVA with Tukey's post-hoc, $p < 0.0001$. **X** indicates time when all mice died or were euthanized within that experimental group. # marks outlier at 60 hours amongst mice fed 2% DSS (n = 2). Number of mice for 0% DSS n = 40, 0.25% DSS n = 26, 1% DSS n = 26, 2% DSS n = 12, 3% DSS n = 6.

Error bars are SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

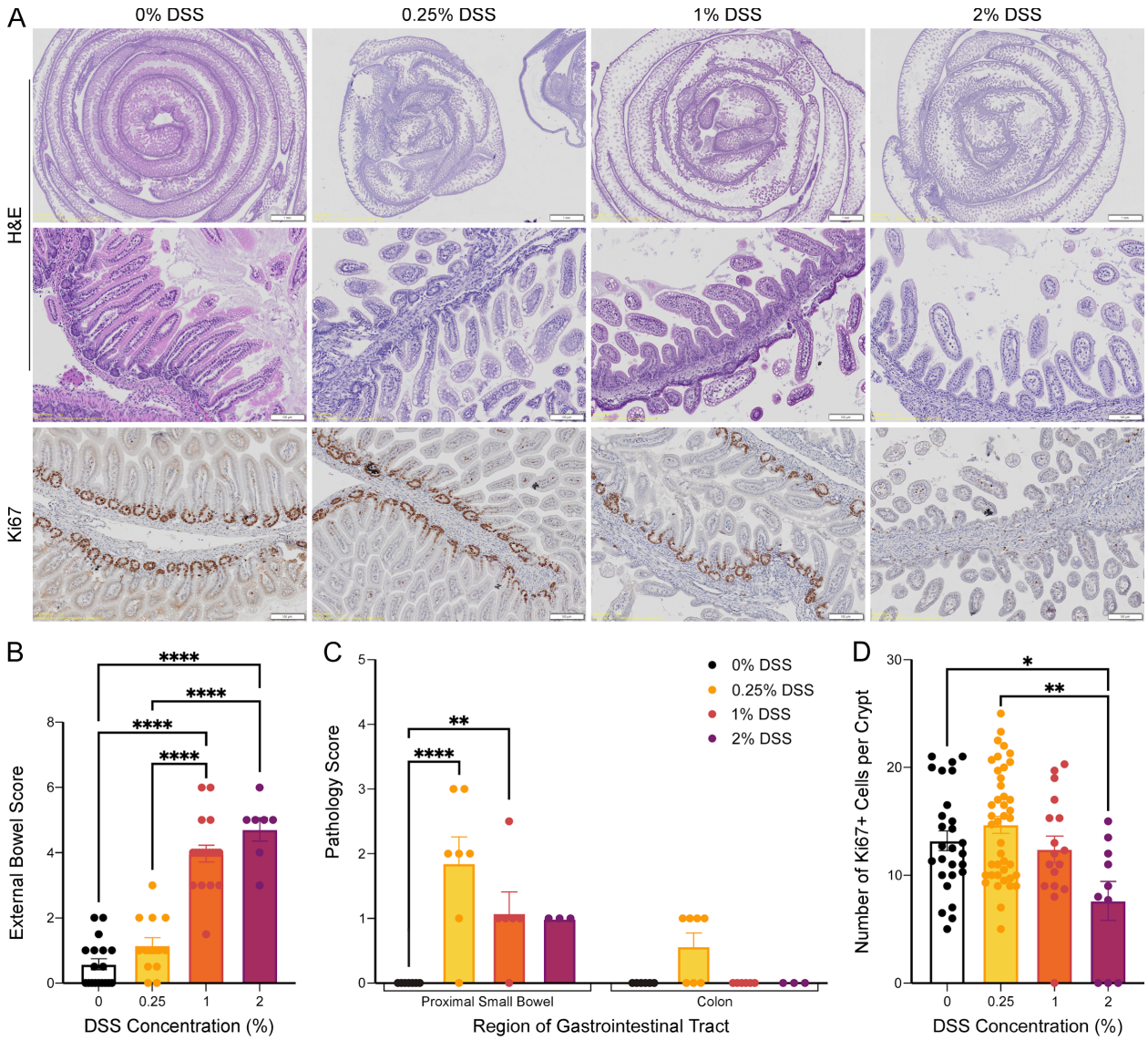


Figure 3. Enterocolitis severity and disorganized gastrointestinal (GI) pathology correlate with DSS concentration.

(A) Representative images of hematoxylin and eosin-stained intestinal samples imaged at 10x (1st row, scale 1 mm) and 200x magnification (2nd row, scale 100 µm). In adjacent slides, Ki67 immunohistochemistry at 200x magnification (3rd row, scale 100 µm). There is greater disorganization and enterocolitis and fewer Ki67+ cells with increasing DSS concentration.

(B) External bowel severity score correlates with increasing DSS concentration. 0% and 0.25% significantly different than 1% and 2%. One-way ANOVA with Tukey's post-hoc, $p < 0.0001$. Number of mice for 0% DSS: n = 18, 0.25% DSS: n = 13, 1% DSS: n = 18, 2% DSS: n = 7.

(C) Pathology score assessment demonstrates that enterocolitis is seen in small bowel more often than colon. Two-way ANOVA with Tukey's post-hoc, $p < 0.0001$. Number of intestines for 0% DSS: n = 7, 0.25% DSS: n = 7, 1% DSS: n = 6, 2% DSS: n = 3.

(D) Cellular proliferation, as measured by Ki67-positive cells within small bowel crypts, decreased significantly in mice fed with 2% DSS compared to mice fed with either 0% DSS or 0.25% DSS. One-

way ANOVA with Tukey's post-hoc, $p < 0.0001$. Number of crypts for 0% DSS: n = 27, 0.25% DSS: n = 42, 1% DSS: n = 16, 2% DSS: n = 7.

Error bars are SEM. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

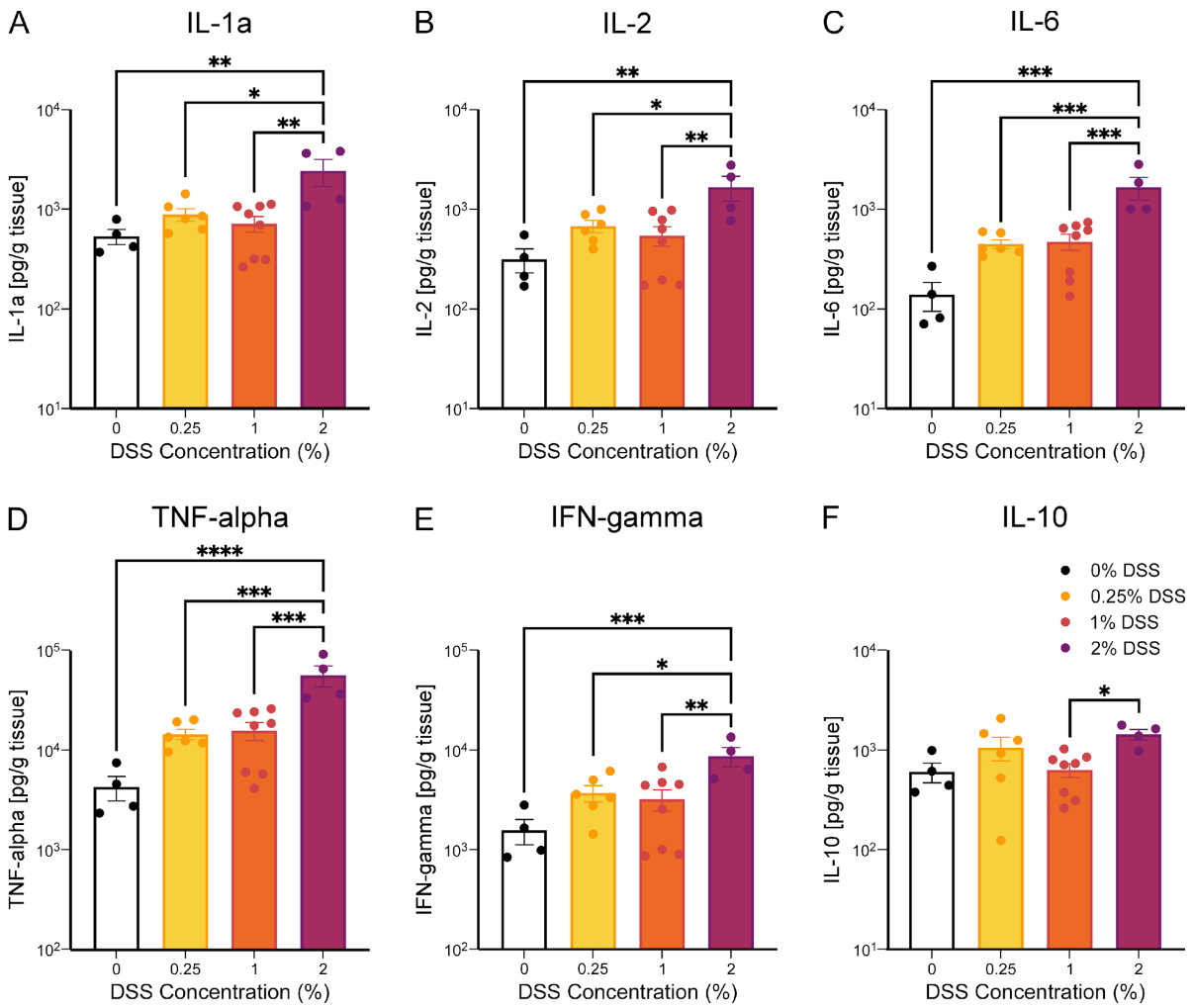


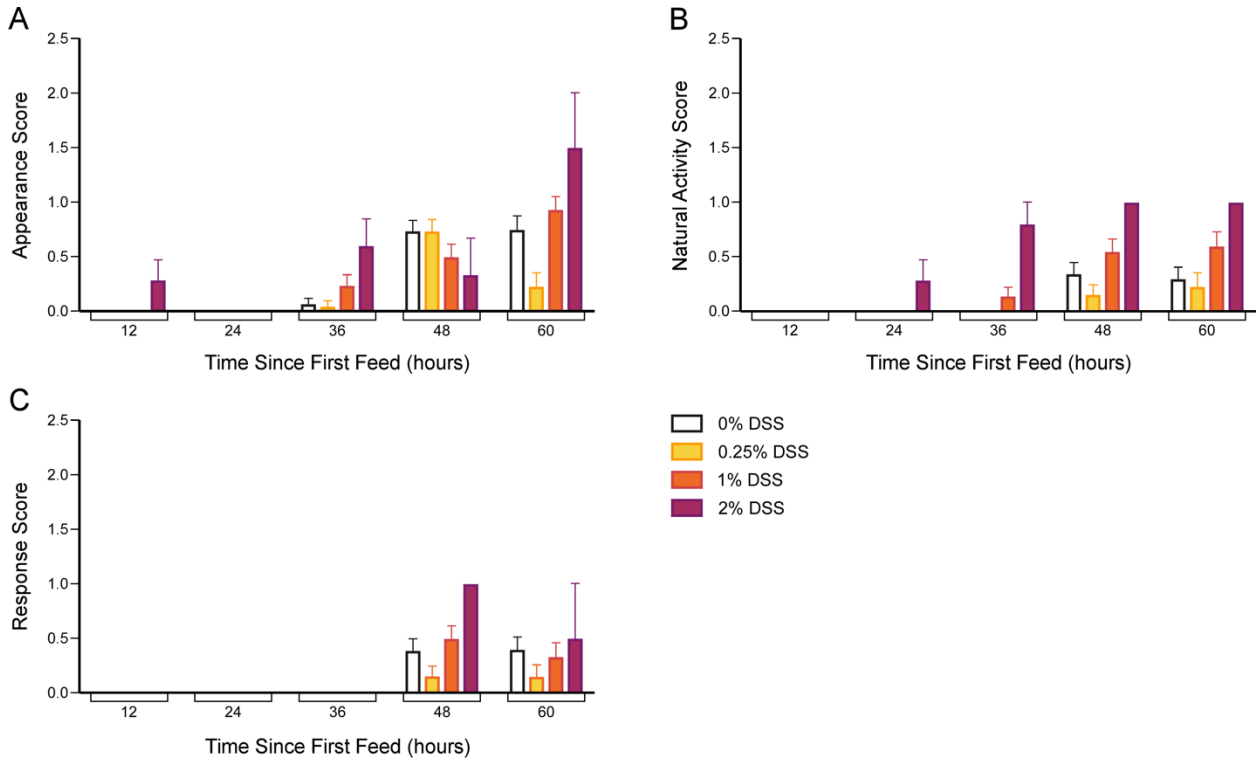
Figure 4. Hepatic pro-inflammatory cytokine concentrations are significantly higher in mice fed 2% DSS.

(A-E) Proinflammatory cytokines **(A)** IL-1α, $p = 0.0022$, **(B)** IL-2, $p = 0.0024$, **(C)** IL-6, $p = 0.0001$, **(D)** TNF-α, $p < 0.0001$, and **(E)** IFN-γ, $p = 0.0017$, are significantly higher in mice fed 2% DSS compared to all other experimental groups. One-way ANOVA with Tukey's post-hoc.

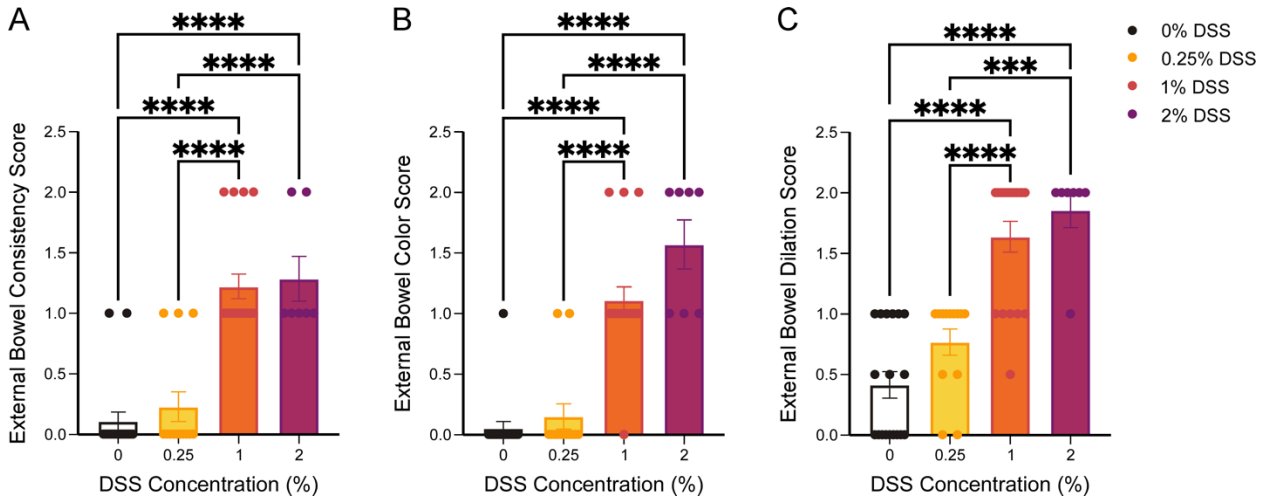
(F) Anti-inflammatory cytokine IL-10 showed little significant difference across all groups. One-way ANOVA with Tukey's post-hoc, $p = 0.0290$.

Error bars are SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Number of livers for 0% DSS: n = 4, 0.25% DSS: n = 6, 1% DSS: n = 8, DSS 2%: n = 4.

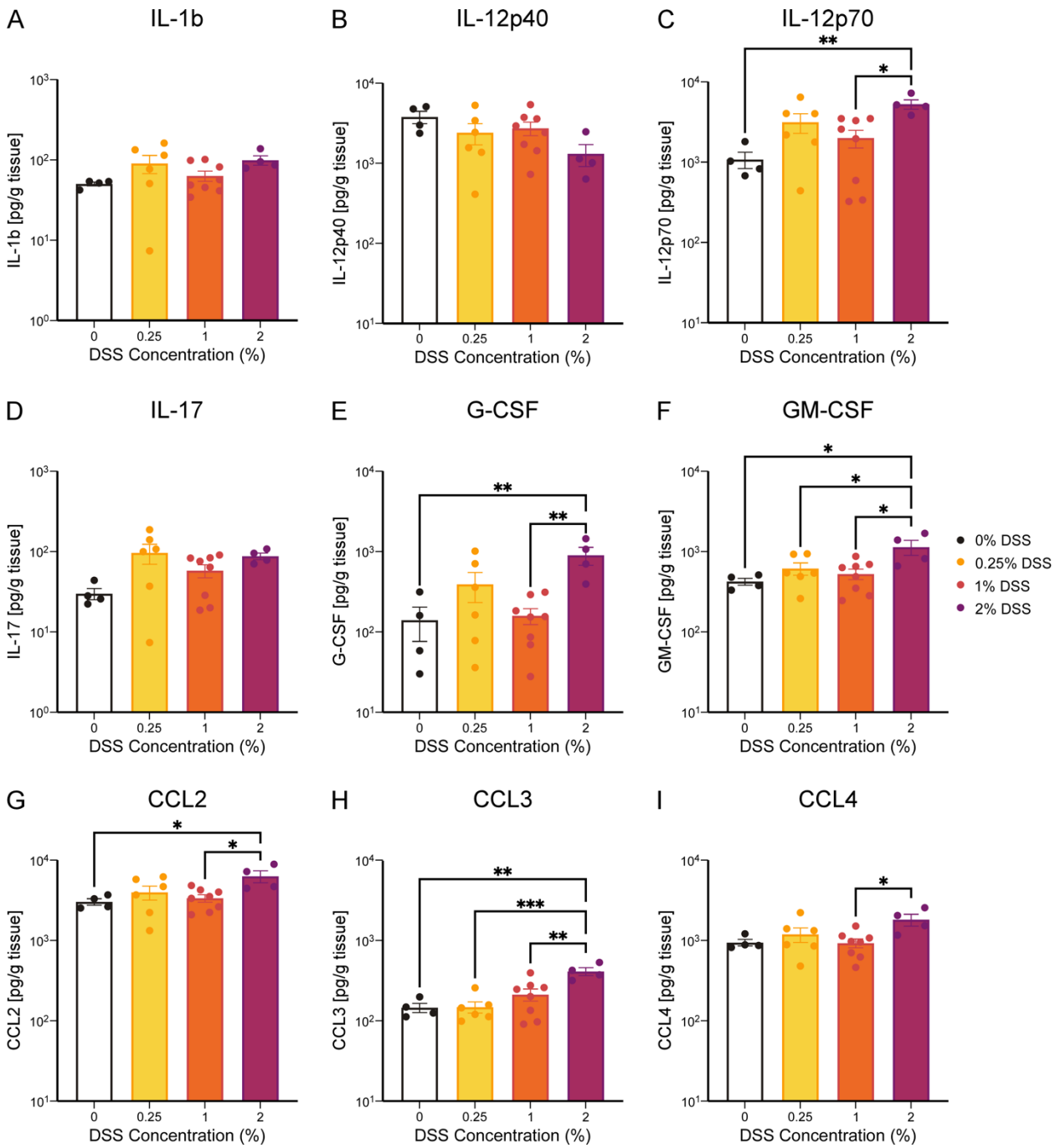
SUPPLEMENTARY MATERIAL



Supplemental Figure 1. Modified Clinical Sickness Score (CSS) based upon Zani et al (2008. Data presented for each score component and stratified by feeding condition. **(A)** Appearance, **(B)** natural activity, and **(C)** response to touch scores increase over the course of the feeding protocol, even when animals are fed formula. Furthermore, higher concentrations of DSS are associated with worse CSS earlier. Two-way ANOVA with Tukey's post-hoc, $p < 0.0001$ for all analyses. Error bars are SEM. Number of mice for 0% DSS: n = 29, 0.25% DSS: n = 26, 1% DSS: n = 26, 2% DSS: n = 7.



Supplemental Figure 2. Macroscopic gut assessment as described in Zani et al (2008)[15]. Bowel **(A)** consistency **(B)** color, and **(C)** dilation is significantly more extensive in mice fed with increasing concentrations of DSS (1% and 2% DSS), compared to lower concentrations of DSS (0% and 0.25% DSS). One-way ANOVA with Tukey's post-hoc, $p < 0.0001$ for all analyses. Error bars are SEM. ***$p < 0.001$, ****$p < 0.0001$. Number of mice for 0% DSS: n = 18, 0.25% DSS: n = 13, 1% DSS: n = 18, 2% DSS: n = 7.



Supplemental Figure 3. Liver cytokines **(A)** IL-1β, $p = 0.1669$, **(B)** IL-12p40, $p = 0.1476$, and **(D)** IL-17, $p = 0.0798$, demonstrate no significant concentration differences across all experimental groups. **(E)** G-CSF, $p = 0.0030$, and **(H)** CCL3, $p = 0.0004$, levels are significantly higher in mice fed 2% DSS compared to all other experimental groups. **(C)** IL-12p70, $p = 0.0055$, **(F)** GM-CSF, $p = 0.0077$, and **(G)** CCL2, $p = 0.0200$, levels are only significantly different when comparing mice fed 2% DSS against mice fed either 0% or 1% DSS. **(I)** CCL4, $p = 0.0296$, levels are only significantly different between 1% and 2% mice. One-way ANOVA with Tukey's post-hoc. Error bars are SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Number of mice for 0% DSS: n = 4, 0.25% DSS: n = 6, 1% DSS: n = 8, 2% DSS: n = 4.