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Early Life Stress and Endothelin Receptor Type A: Separate Roles in Mediating Colitis

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EARLY LIFE STRESS AND ENDOTHELIN RECEPTOR TYPE A: SEPARATE ROLES IN MEDIATING COLITIS

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MICROBIOLOGY

ABSTRACT

Inflammatory bowel disease (IBD) results from the interplay of genetic susceptibility, environmental influences, the microbiota, and aberrant immune responses. Despite the fact that over 200 genetic susceptibility loci have been identified, the genetic contribution to disease onset is believed to be less than 50%. Environmental factors have arisen as key drivers of disease. Early life stress (ELS) is an understudied environmental trigger linked to IBD susceptibility. Utilizing 2 separate models of ELS, we show that ELS results in diminished colonic corticosterone and prolonged intestinal inflammation upon colitogenic insult. ELS-exposed animals consistently demonstrated high levels of colonic *Tnf*, concurrently with decreased expression of genes associated with corticosterone responsiveness and local synthesis. We further establish a connection between the elevated *Tnf* and locally impaired corticosterone, by demonstrating that *ex vivo* TNF stimulation of colonic crypt cells from ELS mice led to enhanced inhibition of corticosterone production. Thus, this study identified impaired local corticosterone synthesis as a potential mechanism whereby ELS predisposes to exacerbated colitis.

Environmental factors also heavily influence the microbiota, with some researchers even considering the microbiota as an internal environmental factor. Endothelial dysfunction and vascular complications commonly manifest in chronic inflammatory conditions, including IBD. The microbiota has been identified as a critical factor in vascular physiology – thus, we investigated the impact of the microbiota on colonic expression of

the endothelin (ET) system. We reveal that the microbiota regulates colonic gene expression of both ET receptors and peptides. Given the importance of the microbiota in IBD pathology, we further assessed the role of the ET system in colitis. We show that prophylactic antagonism of ET receptor type A prevents the development of colitis and maintains colonic epithelial and endothelial barrier integrity. Further, gene expression data from colonic barrier cells indicates that barrier integrity is maintained by preventing colitis-induced alterations in junctional proteins. Collectively, my thesis work reveals an environmental trigger and molecular mediator of colitis and demonstrates potential mechanistic pathways of therapeutic potential related to each.

Keywords: colitis, early life stress, corticosterone, TNF, endothelin system, colonic barrier integrity

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INTRODUCTION

Statement of the Problem

General Overview of the Multifactorial Nature of Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) – including the two main subtypes, ulcerative colitis (UC) and Crohn's disease (CD) – is a multifactorial inflammatory condition of the gastrointestinal tract characterized by a relapsing-remitting clinical phenotype. The exact etiology of IBD is unknown, but it is believed that a combination of genetic predisposition, environmental factors, and dysregulated immune responses to the microbiota all contribute to disease onset[1-3]. The global burden of IBD in 2017 was estimated to be 6.9 million individuals, with the United States comprising nearly 22% of that total at 1.5 million people[4]. Though IBD has been described since the beginning of the 20th century, we still do not have a firm mechanistic grasp as to what leads to IBD onset. Due to the complex, multifactorial nature of this pathology, it is likely that multiple avenues of a different combination of factors can lead to various manifestations of IBD.

Genome-wide association studies (GWAS) have revealed 200+ genetic loci as susceptibility markers for IBD. Many of the identified risk variants are in genes related to epithelial barrier function, microbial peptide recognition, immune tolerance, and innate and adaptive immune responses[5-7]. Despite the ability to identify genetic susceptibility loci, only around 25% of IBD heritability has been described by genetic studies[8]— clearly revealing a role for environmental factors in mediating IBD onset. Though historically

believed to be a disease predominantly affecting white individuals[9], the swift rise in disease incidence around the world over the past 50 years – including in Asian countries, where IBD was once rare[10] – suggests a key environmental role for industrialization, urbanization, and westernization in mediating disease[11]. Societal urbanization is associated with changes in diet, sanitation, antibiotic use, and pollution – all of which have been identified as environmental risk factors for the development of IBD[12,13].

The intestinal microbiota – a group of microorganisms residing in the gut – is believed to exceed 10^{14} cells; this accounts for roughly 10 times the number of human cells and 100 times more genomic content relative to the human genome[14]. Additionally, the coevolution between the microbiota and the human gut has been occurring for millions of years. Thus this consortium of microbes and their genomic content has been viewed by some as a hidden metabolic 'organ', due to the considerable role it plays in human health – including host metabolism, nutrition, physiology and immune development[15,16]. While it is widely accepted that IBD patients exhibit dysregulated microbiotas, it is unclear whether this precedes IBD onset or is a result of the disease[1,17]. Regardless, the inflammatory reaction in IBD is largely driven by exposure to bacterial antigens, upon breach of the epithelial barrier. Disease is often perpetuated by a feed-forward interaction between this dysregulated microbiota and activated immune cells[18-20].

It has become increasingly clear that environmental factors/exposures play a key role in mediating IBD. At the center of the physical, chemical, and immunological intersection between the environment and the host is the gut microbiota[21]. Thus, environmental factors, the microbiota, and human health are intimately connected. Given this, much attention has recently been focused on the exposome and IBD. The exposome

refers to the composite of accumulated environmental exposures in a person's lifetime, beginning in utero[2]. The microbiota is highly sensitive to and influenced by several environmental factors, including but not limited to: diet, pollution, geographic location, smoking, stress, and antibiotic use[1,2,12]. While host genetics partly influence gut microbial structure[22], environmental exposures throughout a person's lifetime will dynamically alter the structure, function, and composition of the intestinal microbiome, thus influencing the risk and natural history of morbidity[23,24].

Though IBD is a lifelong disease with no cure, remission can be gained with the use of therapeutics. Highlighting the immune-driven clinical manifestation of this disease, is the fact that nearly every therapeutic agent to help treat IBD is some form of immune modulator[25,26]. Despite the fact that several therapeutics and biologics exist for treatment of IBD, 40% of patients are primary non-responders to medication and an additional 13-46% of initial responders lose responsiveness within the first year of treatment[27]. Further, extrapolated pharmaco-economic data from 2004 – 2008 to determine cost based off of IBD incidence, revealed that the annual financial burden of IBD in the US ranged from $14.6 - $31.6 billion in 2014[28]. Moreover, a systemic review from 2018 that assessed 64 studies spanning Asia, Europe, and North America from 1985-2018 and cost adjusted for US dollars in 2018, found that the societal cost of illness for IBD is increasing worldwide[29]. This global increase in cost for IBD is largely driven by the already high and increasing cost of biologics[28-30]. Given the correlation between IBD prevalence and industrialized countries, along with the fact that nearly three quarters of all people (3-5 billion) currently live in developing countries, even minor increases in disease incidence could have significant economic ramifications for developing countries[4]. Thus, IBD exhibits a

significant economic burden and is likely only going to continue to increase in financial demands. All of this together – disease complexity, unclear etiology, ≥ 50 % unresponsive or loss of response to therapeutics, and the financial burden associated with disease – point to a continued need to study IBD.

The overarching focus of this thesis explores both environmental risk factors and potential molecular pathways associated with IBD. The work presented in Chapter 2 demonstrates how early life stress (ELS) contributes to IBD susceptibility and severity. We identified reductions in colonic corticosterone synthesis and responsiveness as a potential mechanism whereby ELS mediates sustained colitis in susceptible individuals. Chapter 3 reveals the impact of the microbiota on colonic expression of the endothelin system, as well as the role of endothelin receptor type A in facilitating IBD pathogenesis. We show that prophylactic antagonism of endothelin receptor type A maintains colonic barrier integrity, likely by preventing colitis-induced alterations of junctional proteins.

The Stress Response, Glucocorticoid Signaling, and the Impact of Early Life Stress on Disease

Early Life Stress and Adverse Health Outcomes

According to the 2016 National Survey of Children's Health, 34 million children aged 0 – 17 – almost half of all US children at the time – had experienced one of nine Adverse Childhood Experiences (ACE) and 21.7% had experienced 2 or more ACEs. ACEs are defined as traumatic events that are capable of inducing a toxic stress response which may be detrimental to the long-term health of the child into adulthood[31,32]. The more

ACEs that a person has been exposed to correlates with an increased likelihood for adult adverse health outcomes, both mentally and physically[33,34]. Further supporting this is the fact that early life stress (ELS) has been linked to anxiety, depression, cardiovascular disease, autoimmune diseases, etc.[35-38]. It has been suggested that many adult diseases are actually developmental disorders resulting from biological disruptions occurring early in life during critical stages of plastic development[39-41]. The early postnatal period for all mammals is characterized by pivotal developmental changes in preparation for extra-uterine survival [42]. In addition to brain development[43-46], the immune[47], enteric[48] and nervous systems[49], intestinal epithelial barrier, and microbiota colonization are all subject to plasticity and developmental programming early in life[50,51]. Disruptions – such as stress – to normal maturity in particularly sensitive developmental periods may render an individual more susceptible to diseases and long-term functional issues.

Stress is generally defined as "a real or interpreted threat to the physiological or psychological integrity of an individual that results in physiological and/or behavioral responses"[52]. Allostasis is the overall adaptive reaction of the body, via the hypothalamic-pituitary-adrenal (HPA) axis, to maintain physiological stability (mediated by hormones, neurotransmitters, and the immune system) in response to environmental demands[53,54]. This brain-centered feed forward regulation of the physiological condition in response to a perceived threat is beneficial in the short-term, but is detrimental when these allostatic mediators are sustained in the body, as occurs during chronic stress exposure[55]. Long term stress exposure may cause dysfunction or repression of allostatic mechanisms as a result of excessive activation, termed allostatic load, which can lead to metabolic and immune dysfunction[56]. In fact, Danese et al. followed a cohort from birth through age 32 and found

an association between childhood maltreatment and adult low-grade inflammation (measured by C-reactive protein, fibrinogen, and white blood cell count), independent of co-occurring early life risks, stress in adulthood, and adult health[57].

The current working theories for how ELS predisposes to later life adverse health outcomes are termed the biological embedding hypothesis and the developmental mismatch hypothesis. According to this ideology, stress during sensitive developmental periods can alter how certain physiologic systems operate moving forward. The resultant ELS-induced altered biological programming focuses on epigenetics, tissue remodeling, and post-translational modifications as a means for how stress gets "embedded" or "imprinted" in cells[58]. Further, the developmental mismatch hypothesis points to a mismatch in early and later life environments – biological alterations resulting from stress exposure early in life are beneficial if the later life environment is also stressful, but if the later life environment is not matched with the early life environment then the stress-induced alterations are maladaptive[59].

According to the biological embedding model, ELS imprints a signature in immune cells (including monocytes and macrophages) making them poised for excessive inflammatory reactions and more resistant to inhibitory signals. A study by Miller et al. utilizing genome-wide transcriptional profiling on peripheral blood mononuclear cells (PBMCs) from adults who were either low or high socioeconomic status (SES) early in life seems to corroborate the biological embedding model of ELS. They observed a significant down regulation of genes with response elements for the glucocorticoid receptor and significant upregulation of genes with response elements for nuclear factor κB (NFκB) in adults from a low-SES early life background compared to adults from a high-SES

background. Importantly, these results were independent of the subject's current SES and perceived stress[60]. Despite the fact that ELS is associated with a more aggressive inflammatory response, children exposed to stress early in life are actually more susceptible to infection and illness than those with no stress exposure early in life[61]. ELS is associated not only with a greater risk of chronic and autoimmune disorders, but also with acute illness – strongly suggesting that ELS-exposed individuals exhibit immune dysregulation.

Hypothalamic-Pituitary-Adrenal (HPA) Axis: The Body's Main Stress Response System

Humans and animals respond to stress by activating neurons that regulate autonomic and neuroendocrine responses. These self-regulating processes are meant to return essential physiologic systems to a set point within a certain range of operation that ensures survival[62]. To date, the HPA axis is probably the most studied component of the stress response. Upon a perceived stressor, neurons in the paraventricular nucleus (PVN) of the hypothalamus release corticotropin-releasing factor (CRF) and argenine vasopressin (AVP) into blood vessels connected to the pituitary gland. These neuro-hormones stimulate the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) into the circulation. The ACTH then stimulates the adrenal glands (located above the kidney) to produce and secrete glucocorticoids (GCs) – cortisol in people, corticosterone (CORT) in rodents[63].

Glucocorticoids are essential for energy regulation in the body to meet metabolic demand, largely through regulation of glucose availability[64]. Due to the highly important role of GCs in multiple physiologic processes, the HPA-axis is carefully modulated through GC-depended negative feedback loops designed to return hormone levels to homeostatic

set points. CORT signals through the mineralocorticoid receptor (MCR, encoded by *NR3C2*) and the glucocorticoid receptor (GCR, encoded by *NR3C1*), both of which are ubiquitously expressed and involved in the negative feedback control of CORT production. The MCR has a higher binding affinity for CORT than the GCR and under homeostatic conditions the MCR regulates the circadian rhythm of circulating CORT. For example, when CORT concentrations are high due to stress and MCRs are saturated, CORT will then bind to the lower affinity GCR and terminate the HPA-axis stress response[62,64,65]. This internal regulatory feedback loop is critical for maintaining physiologic homeostasis, as either too much or too little exposure to CORT can result in severe health consequences.

Beyond neuronal signals, pro-inflammatory cytokines such as IL-1β, IL-6 and TNF can also activate the HPA axis[64]. Additionally, circulating concentrations of GCs are regulated by corticosteroid-binding globulin (CBG) which binds up to 90% of released GCs. CBG helps to systemically distribute CORT, but may also be involved in tissue-specific GC delivery and distribution. Once released from CBG, GCs can freely diffuse through cell membranes to exert function. However, the actual cytoplasmic bioavailability of GCs is controlled by the balance between active and inactive GCs – regulated by two isoforms of 11β-hydroxysteroid dehydrogenase (11β-HSD). 11β-HSD-1 is responsible for the conversion of GC precursors into biologically active GCs whereas 11β-HSD-2 controls the conversion of biologically active GC into inactive GC. Interestingly, these enzymes are also influenced by pro-inflammatory cytokines during an acute inflammatory response; TNF and IL-1β induce 11β-HSD-1 upregulation and 11β-HSD-2 downregulation in multiple cell types, promoting active GCR signaling and subsequent dampening of inflammation[64,65].

Immunomodulatory Outcomes of Signaling through the Glucocorticoid Receptor

As previously mentioned, GCs and the correct regulation of GC levels in the body, are critically important due to the multiple key physiologic systems impacted by GC signaling, and the ubiquitous expression of GC-binding receptors – MCR, GCR. GCs are vital for daily function as they play a role in reproduction, development, metabolism, growth, water and electrolyte balance, cardiovascular function, mood and cognitive function, and the immune system[65]. GCs mediate potent immunomodulatory effects. The extraordinary anti-inflammatory capabilities of GCs is evident by the fact that GCs are the most widely prescribed class of drugs worldwide due to their efficient treatment of acute and chronic inflammation[66].

GCR mediated action involves DNA-binding genomic interactions as well as rapid non-genomic signaling. The GCR can induce anti-inflammatory gene transcription by binding to glucocorticoid response elements (GREs) in regulatory regions; on the other hand, the GCR can bind to negative regulatory GREs and repress pro-inflammatory gene expression[67]. The GCR can also regulate gene expression through protein – protein interactions, or tethering; exemplified by GCR transrepression of key pro-inflammatory transcription factors, NFκB and activator protein-1 (AP-1). Additionally, the GCR can be post-transcriptionally modified – affecting the subcellular localization, stability, interaction with other proteins, and the transcriptional activity of the GCR[68]. Further exhibiting the physiologic importance of GCR signaling is the fact that activated GCR can regulate up to 20% of the genome[65]. Moreover, the complexity and pleiotropy of GCR signaling is further highlighted by several chromatin immunoprecipitation sequencing (ChIPseq) analyses

which found limited overlap of GCR binding sites between different cell types and tissues – indicating cell-type specific GCR-mediated action[68].

Intestinal Glucocorticoid Synthesis

While circulating GCs are mainly produced by the adrenal glands, a number of other organs have been found to produce bioactive GCs – including the thymus, intestine, lung and skin. Extra-adrenal *de novo* GC synthesis is believed to act locally in a paracrine matter, and the contribution of extra-adrenal GCs to the circulation is negligible[69]. Supporting this notion of local paracrine signaling is the fact that intestinal GC synthesis is differentially regulated from adrenal GC synthesis. In the adrenal glands, ACTH induces and activates steroidogenic factor-1 (SF-1, encoded by *NR5A1*) which is critically involved in the regulation of adrenal GC synthesis[70]. While SF-1 is abundantly expressed in the adrenals, it is absent in the intestine and is replaced with its close homolog, liver receptor homolog-1 (LRH-1, encoded by *NR5A2*) for intestinal-derived GC synthesis[71]. Additionally, ACTH receptor activation induces cyclic adenosine monophosphate (cAMP), which is critical for adrenal steroidogenic enzyme induction. Adrenal cell treatment with cell-permeable cAMP induces strong steroidogenic gene expression, but the treatment of intestinal epithelial cell lines with cAMP actually inhibits Lrh-1-mediated intestinal steroidogenesis[72]. HSD enzymes involved in the interconversion of biologically active and inactive GC are also expressed in the intestines. The expression of 11βHSD-1 (converts inactive CORT to active CORT) in lymphoid tissue is upregulated in response to inflammatory cytokines and GCR activation to help control inflammation[73]. Thus,

intestinal GC levels are locally regulated, and this is at least partially independent of the HPA-axis.

Activation through LRH-1 is responsible for mediating intestinal GC synthesis. Intestinal epithelial cells (IECs), specifically crypt IECs were found to express LRH-1 and thus produce and secrete local GCs. As IECs mature and move up the crypts, LRH-1 expression is lost[74]. In addition to its role in *de novo* GC synthesis, LRH-1 is also involved in epithelial cell renewal and cell cycle progression. LRH-1 is involved in intestinal cell renewal by synergizing with the β-catenin pathway to regulate cyclin D1, cyclin E1, and c-Myc[75]. Thus, LRH-1 contributes to maintenance of intestinal homeostasis via the production of anti-inflammatory GCs to limit/ameliorate inflammatory immune cell-induced damage and may also accelerate repair/recovery of damaged epithelium by increasing proliferation of intestinal epithelial stem cells in the crypts. Additionally, local intestinal GCs are also likely involved in maintaining epithelial barrier integrity. In fact, microarray data from hydrocortisone-treated human IECs showed regulation of protein-encoding genes and enterocyte markers involved in tight junction formation and polarization[76].

LRH-1 is technically considered an orphan nuclear receptor, though phospholipids have been found to bind with this receptor. The connection between intestinal GCs and immune regulation is further evidenced by the fact that activation of both the innate and adaptive immune system – via intraperitoneal (IP) injections of lipopolysaccharide (LPS) or anti-CD3, respectively – induce robust expression of intestinal Lrh-1 and downstream steroidogenic enzymes[71,77]. The importance of LRH-1 in mediating intestinal homeostasis is further highlighted by the fact that GWAS analyses have identified a strong association

between LRH-1 and IBD[6,78]. Animal studies using Lrh-1 haploinsufficient mice or IEC specific knockout of Lrh-1 observed dysregulated epithelial proliferation and increased susceptibility to colitis[75,79]. Moreover, colon tissue biopsies from IBD patients exhibited reduced expression of LRH-1 and downstream steroidogenic enzymes. Further, these results were inversely correlated with pro-inflammatory cytokine expression[79]. A recent study found that LRH-1 overexpression alleviated inflammation-induced damage of murine and human intestinal organoids, including those derived from IBD patients. Further, this LRH-1 overexpression significantly reduced disease severity in a T cell transfer model of colitis[80]. Given this established connection between IBD and reduced LRH-1, along with the knowledge that LRH-1 controls intestinal steroidogenesis, positions LRH-1 as an attractive investigative target to study interactions between ELS and IBD pathogenesis.

Implications of Stress and IBD

A relationship between stress and IBD has been realized for decades, especially given that IBD was considered a psychosomatic disorder in the 1950s[81]. No longer believed to be the case, the fact remains that stress represents a key environmental trigger in IBD disease flares and exacerbations[82,83]. Further, psychiatric comorbidity is 3 times higher in IBD patients than the general population[84]. Interestingly, a population-based study conducted in Manitoba, Canada found that 159 out of 351 study participants had a lifetime anxiety or mood disorder and within this subset, 79% of individuals reported that the first symptoms of anxiety disorder predated their IBD diagnosis by more than 2 years[85]. These observations have led to much postulation about potential common pathways between

psychiatric illnesses and chronic inflammatory conditions, with particular focus on dysfunctional immune-regulatory circuits[86].

Early life stress specifically has also been associated with an increased risk of IBD. Childhood neglect, with an emphasis on attachment style and low parental care, has specifically been associated with IBD susceptibility[87-89]. Further, a population-based study with nearly 22,000 participants found that those who had experienced childhood sexual and physical abuse displayed 3.3 and 2.18 times the odds respectively, of also having ulcerative colitis[90]. Additionally, studies utilizing various models of IBD have demonstrated that ELS enhances susceptibility to colitis, alters barrier function, and increases pro-inflammatory gene expression[91-93]. Despite these observations, the mechanism of action remains unclear. Chapter 2 of this thesis explores a potential mechanistic pathway whereby ELS mediates prolonged intestinal inflammation.

The Endothelium in Health and Disease: A Focus on the Endothelin System

Introduction to the Endothelin System and the Endothelium

The endothelin (ET) signaling peptides (ET-1, -2, -3) are made up of 3 structurally similar 21-amino acid sequences. The ET peptides signal through one of two G-protein coupled receptors (GPCRs), endothelin receptor type A (ET_A, encoded by *EDNRA*) and endothelin receptor type B (ET_B, encoded by *EDNRB*). ET-1 and ET-2 bind the endothelin receptors with equal affinity, whereas ET-3 has lower affinity for ET_A[94]. Biologically active ETs are regulated at the level of transcription; ET precursors are processed sequentially by 2 proteases to generate the biologically active form. Preproendothelins are

cleaved by furin-like endopeptidase to generate biologically inactive intermediates, termed big endothelins. The mature, biologically active endothelin is generated when big ETs are then cleaved by endothelin-converting enzymes (ECEs), a family of membrane-bound zinc metalloproteases[95].

The endothelium is a single layer of cells making up the inner layer of all blood vessels and the lymphatic system. Once believed to be inert, the endothelium is now appreciated as a dynamic endocrine organ[96]. The most widely recognized function of the endothelium is maintenance of vascular tone, but the endothelium also functions in blood fluidity, barrier integrity, regulation of cellular and nutrient trafficking, contributes to the local balance of inflammatory mediators, participates in angiogenesis, and contributes to procoagulant and anticoagulant activity[96,97]. Given the numerous functions and widespread distribution of the endothelium, it is fairly unsurprising that endothelial cells exhibit significant phenotypic heterogeneity by location and signaling cues[98]. Endothelial cell heterogeneity results from both intrinsic factors, such as genetics, and extrinsic factors such as location, cell-to-cell contact, cell-matrix interactions, soluble mediators, pH, oxygen, and mechanical forces[99].

Highlighting the phenotypic heterogeneity, endothelial cells exhibit differential structural integrity based on location. Endothelial cells may be continuous with or without fenestrae or discontinuous. Fenestrated continuous endothelium are present in sites where increased filtration or increased transendothelial transport are common. The intestinal endothelium is characterized by diaphragm-covered fenestrae. Fenestrae are transcellular pores that allow for increased permeability, though the increased permeability is restricted

to water and small molecules under homeostatic conditions, due to the presence of a size-selective/size-limiting diaphragm[100].

The endothelial barrier is maintained by intercellular junctions, namely adherens junctions and tight junctions, which regulate molecular and cellular transport between the circulation and underlying tissue. Increased vascular permeability is characterized by the detachment of endothelial adherens junctions at focal regions and intercellular gap formation[101]. Plasmalemma vesicle-associated protein (PV-1, encoded by *PLVAP*) is an endothelial-specific protein that is critical for vascular homeostasis. PV-1 forms the stomatal and fenestral diaphragms of the endothelium and helps to regulate angiogenesis, basal permeability, and leukocyte migration[102]. Highlighting the importance of PV-1, Stan et al. demonstrated that genetic deletion of PV-1 in mice resulted in premature death from severe edema, with the highest detection of protein leakage found within the intestine[103].

Activation of ET_A or ET_B on vascular smooth muscle cells (VSMCs) results in vasoconstriction whereas activation of ET_B on endothelial cells results in vasodilation. While a number of molecules mediate regulatory effects on vascular tone, the key players involve a critical balance between ET-1 and nitric oxide (NO)[104]. This dynamic relationship is evidenced by the fact that endothelial-secreted ET-1 will signal in an autocrine manner to endothelial ET_B to stimulate the release of NO – acting as an important feedback mechanism to limit ET-mediated constriction[94].

Endothelial Dysfunction and Chronic Inflammation

Endothelial dysfunction arises due to "an imbalance between vasodilating and vasoconstricting substances produced by (or acting on) endothelial cells"[105]. While an imbalance of any of the vasoactive substances may result in endothelial dysfunction, the ET-1/NO axis is generally regarded as the most critically relevant for the induction of endothelial dysfunction. Endothelial dysfunction is characterized by compromised barrier function, upregulation of cellular adhesion molecules, increased leukocyte diapedesis, and increased vascular smooth muscle tone[106].

Several chronic inflammatory diseases are associated with endothelial dysfunction and an increased risk of cardiovascular complications, including: rheumatoid arthritis, psoriasis, systemic lupus erythematosus, the seronegative spondyloarthropathies, periodontitis, atopic dermatitis, and IBD[106-108]. In addition to the obvious shared characteristic of chronic inflammation, oxidative stress is another common feature amongst these conditions. Oxidative stress and inflammation are important underlying mechanisms driving endothelial dysfunction[109]. Thus, vascular complications and cardiovascular co-morbidity in chronic inflammatory diseases are believed to manifest secondarily as a result of the oxidative stress and inflammatory features of these chronic conditions. Further, the multi-directional effects of oxidative stress, inflammation, and endothelial dysfunction on one another can lead to a vicious feed-forward cycle that sustains pathophysiology[107,108].

The Intestinal Vasculature

The central function of the gastrointestinal (GI) tract is to digest and absorb ingested food and water. While the intestinal epithelium mediates the extraction of nutrients from the lumen, blood and lymph vessels are responsible for delivering the absorbed nutrients and water throughout the body[110]. Additionally, the endothelin system regulates intestinal peristalsis; ET_A receptor activation stimulates intestinal movement whereas ET_B receptor activation inhibits intestinal peristalsis[111,112].

The small and large intestine receive blood supply via the celiac, superior, and inferior mesenteric arteries. In the resting state, these 3 vessels receive 20-25% of cardiac output, but following a meal, intestinal blood flow can double[113]. Blood flow within the GI tract is not uniform; the mucosal layer receives 70-80% of the blood supply, depending on food intake[110]. The GI tract exhibits a unique vascular network; a vertical oxygen gradient from the anaerobic lumen through the epithelium to the highly vascularized mucosa has been recognized in the colon[114]. This unique vascular network results in low oxygenation in the most luminal areas of the intestine, termed physiologic hypoxia. This physiologic hypoxia makes the intestine particularly sensitive to microcirculatory disturbances[113,115].

Vascular Complications in IBD: A Role for the Endothelin System?

The colonic microvasculature in affected tissues of IBD patients exhibit different structural and functional changes within all segments of the vascular tree[116]. Colonic blood flow alterations have been detected in IBD patients as well as in animal models of colitis. Early stages of IBD are characterized by significant increases in blood flow, whereas later

fibrotic stages of disease exhibit below average intestinal perfusion[117-119]. The elevated angiogenesis at affected tissue sites observed in IBD is a hallmark of chronic inflammation. Angiogenesis at sights of inflammation is necessary for the delivery of nutrients and oxygen to the injured area, removal of waste products, and cellular chemotaxis. Angiogenesis becomes pathologic during chronic inflammation[120]. In fact, angiogenesis of affected tissue is correlated with IBD severity[121,122].

A recent meta-analysis assessing the relationship between IBD and cardiovascular disease found that IBD patients exhibited significant endothelial dysfunction, arterial stiffness and carotid intima-media thickness compared to matched controls[123]. Endothelial dysfunction has even been demonstrated in pediatric IBD patients, with one study detecting endothelial dysfunction at diagnosis[124,125]. Further, Garolla et al. demonstrated that the number of circulating endothelial precursor cells, which are considered markers of vascular healing, were significantly decreased in IBD patients compared with healthy controls[126]. The ET system has previously been linked to IBD pathogenesis. Plasma ET-1 is increased in CD and UC patients[127] and elevated endothelin reactivity has been detected in inflamed tissue biopsies of IBD patients[128-130]. Moreover, it has previously been demonstrated that ET receptor antagonists prevent mucosal damage in rodent studies utilizing chemically-induced models of colitis[131-133]. Despite these observations, the mechanism by which ET receptor antagonism mediates protection from intestinal pathology remains unclear. Chapter 3 will explore the impact of the microbiota on colonic expression of the ET system, in addition to a potential mechanism whereby ET_A antagonism facilitates protection from colitis.

EARLY LIFE STRESS IN MICE LEADS TO IMPAIRED COLONIC CORTICOSTERONE PRODUCTION AND PROLONGED INFLAMMATION FOLLOWING INDUCTION OF COLITIS

by

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Inflammatory Bowel Diseases

Format adapted and errata corrected for dissertation

Summary:

Using two distinct animal models, this study shows that in mice, early life stress leads to reduced colonic corticosterone and that induction of colitis after stress removal results in reduced transcription of glucocorticoid synthesis genes, increased *Tnf*, and enhanced chronicity of intestinal inflammation.

Running Title: Early life stress and susceptibility to colitis

ABSTRACT

Background Early life stress (ELS) is an environmental trigger believed to promote increased risk of IBD. Our goal was to identify mechanisms whereby ELS in mice affects susceptibility to, and/or severity of, gut inflammation.

Methods: We utilized 2 published animal models of ELS. In the first model, newborn mice were separated from the dam daily for 4-8 hours starting on postnatal day 2, then weaned early on postnatal day 17. Control mice were left undisturbed with the dams until weaning on postnatal day 21. In the second model, dams were fed dexamethasone or vehicle ad libitum in drinking water on post-partum days 1-14. Plasma and colonic corticosterone were measured in juvenile and adult mice. Colitis was induced in 4-week-old mice via intraperitoneal injection of IL-10 receptor blocking antibody every 5 days for 15 days. Five or 15 days later, colitis scores and transcripts for *Tnf*, glucocorticoid receptors, and steroidogenic enzymes were measured.

Results: ELS-exposed mice displayed reduced plasma and colonic corticosterone. Control animals showed improvements in indices of inflammation following cessation of IL-10 receptor blockade, whereas ELS-exposed animals maintained high levels of *Tnf* and histological signs of colitis. In colitic animals, prior exposure to ELS was associated with significantly lower expression of genes associated with corticosterone synthesis and responsiveness. Finally, TNF stimulation of colonic crypt cells from ELS mice led to increased inhibition of corticosterone synthesis.

Conclusions: Our study identifies impaired local glucocorticoid production and responsiveness as a potential mechanism whereby ELS predisposes to chronic colitis in susceptible hosts.

Keywords: Early life stress, glucocorticoids, TNF, chronic colitis.

Key Messages:

What is already known? ELS can lead to reduced hypothalamic-pituitary-adrenal (HPA) axis function and predispose to increased severity of systemic inflammatory disease.

What is new here? ELS inhibits intestinal production of, and sensitivity to, corticosterone and predisposes to enhanced chronicity of colonic inflammation that is induced after stress exposure.

How can this study help patient care?Considering the multiple stress signals encountered during the formative years of life, an improved understanding of the mechanisms whereby ELS impacts gut immune regulatory cascades can ultimately enhance our ability to predict and/or manage the effects of ELS in IBD-susceptible individuals.

INTRODUCTION

Inflammatory bowel disease (IBD) develops at the intersection of genetic susceptibility and dysregulated immune responses to the microbiota and other environmental triggers. There is still no cure for IBD, but consistent with the elevated levels of tumor necrosis factor (TNF) in affected tissue of IBD patients and the role of this cytokine as a dominant inflammatory mediator of IBD[1], some of the most effective therapies for induction of remission are neutralizing antibodies against TNF[2,3]. Environmental triggers of inflammation, including various forms of psychological stress, are arguably the least understood contributing factors despite increasing acknowledgement of the potential of these stimuli to impact IBD development and/or relapse. Acute psychological stress has long been acknowledged to exacerbate symptoms and trigger relapses in IBD patients[4-6]. Moreover, epidemiological studies retrospectively identified significant positive correlations between IBD diagnosis and adverse childhood experiences (ACEs) – traumatic experiences that occur prior to adulthood[7]. In 2019, the U.S. Centers for Disease Control and Prevention (CDC) reported that of 100,000 adults studied across 25 states, 1 in 6 had experienced 4 or more types of ACEs and that 5 of the top 10 leading causes of death are associated with ACEs[8]. Considering the rates of diagnosis of IBD in children and adolescents, and the multiple potential stress signals encountered during early life, an improved understanding of the mechanisms whereby ELS impacts gut immune regulation in health and during inflammation can ultimately enhance our ability to predict and/or manage the effects of ELS in IBD-susceptible individuals.

The main stress response system of the body is the hypothalamic-pituitary-adrenal (HPA) axis, and it is known that ELS can lead to HPA-axis dysfunction[9,10]. Upon receipt of a perceived stressor, the paraventricular nucleus of the hypothalamus secretes corticotrophin-releasing factor (CRF) and arginine vasopressin (AVP). CRF and AVP stimulate the anterior pituitary gland to secrete adrenocorticotropic hormone (ACTH) which then activates the adrenal glands to release glucocorticoids (GCs) – cortisol in humans and corticosterone (CORT) in rodents[10]. GCs can negatively feedback into the HPA-axis in a dose- and duration-dependent manner to repress the HPA-axis-mediated stress response, including GC production[11]. This controlled regulation of HPA-axis activity is crucial since excessive or diminished GC release can have dire metabolic and immunological consequences[12,13].

Systemic GCs are derived from adrenal glands and can impact multiple physiological processes[13,14] but other organs, including the intestine, are also capable of producing GCs[15]. Intestinal GCs are synthesized by crypt epithelial stem cells following activation of liver receptor homolog-1 (LRH-1), which transcriptionally regulates the expression of steroidogenic enzymes including CYP11A1 and CYP11B1[16]. GCs can signal to multiple cell types due to the ubiquitous expression of the GC receptor (GCR, encoded by *Nr3c1*). Under homeostatic conditions, colon-derived GCs promote maturation and preservation of the epithelial barrier and being potent immune suppressors, help regulate immune cell activation and proliferation[17,18]. Gut-specific GC synthesis has also been implicated in IBD pathogenesis as transcriptome-wide association studies have identified *NR5A2* (which encodes LRH-1) as a susceptibility gene for both Crohn's disease and ulcerative colitis[19]. Compared to healthy tissue, inflamed colonic biopsies from UC and

CD patients exhibit significantly lower levels of *NR5A2*, *CYP11A1*, and *CYP11B1*[20]. Owing to their anti-inflammatory capabilities, administration of synthetic GCs is common practice for the treatment of several inflammatory diseases including IBD[21]. Furthermore, therapeutic targeting of colonic GC production successfully impeded experimental IBD and consequently, investigation into the potential clinical use of LRH-1 agonists is ongoing[22].

Altogether, current knowledge suggests that down-regulation of GC output due to chronic stress can directly and profoundly impact intestinal immune homeostasis and local control of microbiota-dependent inflammation when it arises. Therefore, our goals in this study were to examine (1) whether and how ELS might cause local disruptions in intestinal immune homeostasis, and (2) the potential impact of ELS on the subsequent development and progression of induced colitis. We first employed an established murine model of ELS based on maternal neglect – maternal separation with early weaning (MSEW). In this model, ELS resulted in impaired production of colonic corticosterone and reciprocally skewed expression of anti-inflammatory interleukin-10 (IL-10) and pro-inflammatory interferon-gamma (IFNγ) by mucosal CD4 T cells. When subjected to induction of chronic colitis via transient blockade of the IL-10 receptor, ELS mice displayed increased chronicity of disease characterized by sustained expression of *Tnf* and damage to the epithelial barrier. In a separate model of ELS involving direct HPA axis disruption via postnatal feeding of the synthetic glucocorticoid dexamethasone (Dex), we observed similarly impaired colonic CORT as in MSEW mice, and a similar chronic disease phenotype following introduction of a colitogenic insult. In pre-colitic ELS mice, and despite the lower levels of colonic corticosterone, we observed no differences in expression

of *Nr5a2* compared to control animals. However, the chronic phase of colitis was associated with significant downregulation of *Nr5a2* and the Lrh-1 transcriptional target, steroidogenesis enzyme *Cyp11a1*. Conversely, colonic crypt cells which are the primary source of colonic CORT, harbored increased TNF receptor transcripts and TNF stimulation suppressed GC output of colonic crypt cells from ELS mice, more so than cells enriched from control mice. Collectively, our results demonstrate that ELS enhances the inflammatory tone of the intestine and predisposes to further suppression of intestinal GC synthesis during chronic intestinal inflammation.

MATERIALS and METHODS

Mice

All mice used in this study were on the C57BL/6 genetic background, originally acquired from Jackson Laboratories (Bar Harbor, ME). In initial studies, we observed substantial variability in stress response phenotypes and disease outcomes in female mice that was not evident in males, reminiscent of our previous findings that MSEW promoted endothelial dysfunction in males, but not female mice[23]. Therefore, the results presented herein were generated using male mice. All mice were bred and maintained under specific pathogen-free (SPF) conditions at the University of Alabama at Birmingham in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines.

Maternal Separation with Early Weaning (MSEW)

MSEW was performed as previously described[23,24]. Briefly, pregnant females were monitored and randomly assigned to either normal rear (NR) or MSEW groups. To minimize 'cage effects' on the microbiota each dam underwent two breeding cycles with alternating assignments to NR or MSEW, i.e., dams with normally reared control litters in the first breeding round had litters assigned to the MSEW group in the second breeding round, and vice versa. Exact day of birth was noted as postnatal day 0 (PD0). MSEW litters were separated from dams for 4 hours/day on PD2-5 from 0900h to 1300h. From PD6-16, MSEW pups were separated for 8 hours/day from 0900h to 1700h. MSEW pups were weaned on PD17 with ad libitum access to food and water. During the separation period, pups were housed in an Animal Intensive Care Unit (AICU) incubator (Lyon Technologies,

Catalog No. 912-062), at 37.5°C and 60% humidity. Normally reared (NR) litters remained undisturbed with dams until standard weaning on PD21. All mice were maintained on standard chow (NIH-31; NSN 8710-01-005-8438) following weaning. After weaning, females were rested for a minimum of 7 days prior to being mated again.

Dexamethasone administration

To promote its solubility in water, dexamethasone (D9184, Sigma) was first suspended in vehicle solution comprised of 0.002% DMSO (BP231, Thermo Fisher) and 0.02% 2-hydroxypropyl-b-cyclodextrin (H107, Sigma). Dexamethasone was delivered in the drinking water from PD 1-14 at a final concentration of 3.0 µg/ml. An equal volume of vehicle solution was diluted in water and provided to control animals. Pregnant females were monitored so that exact day of birth could be noted as PD0. Vehicle or dexamethasone was administered ad libitum in drinking water from PD1-14. Fresh preparations were provided every 5 days and all cages were returned to normal drinking water on PD14.

Assessment of Intestinal Permeability

Four-week-old mice were fasted for 6 h before being administered 4-kDa FITC dextran (Sigma-Aldrich, 400 mg/kg body weight) via oral gavage. Four hours after gavage, blood was collected via the retro-orbital plexus into heparinized tubes and plasma was separated by centrifugation at 10,000 g for 10 minutes at room temperature. Plasma was diluted 1:4 in sterile deionized (DI) water in a 96-well plate. Fluorescence was detected by a Synergy HT spectrophotometer (Biotek; excitation: 485 nm, emission: 528 nm). FITC dextran was dissolved in sterile DI water at final concentrations ranging from 0 - 10,000 ng/ml and used to calculate FITC dextran concentrations of the samples. Fluorescent emission signals in

the plasma of mice that did not receive FITC-dextran were averaged and subtracted from emission signals of mice treated with 4-kDa FITC dextran to eliminate background signal.

Tissue dissociation and flow cytometry

Mesenteric lymph nodes and/or colons were collected from mice between 8:00 am – 1:00 pm and were mechanically dissociated in RPMI, and the cell suspension was filtered through a 70 μM mesh strainer. Mesenteric fat was removed from colons, colons were flushed with sterile PBS and cut open longitudinally. Tissue was sectioned into 1 cm pieces and incubated for 20 minutes at 37 °C with rotation in 154 μg/L $_{L}$-dithioerythritol and 2 μM EDTA in HBSS to remove the mucus and epithelial cells. The tissue was then incubated at 37 °C with rotation in 20 μg/mL DNase-I and 100 U/mL collagenase IV (Sigma-Aldrich), and tissue digestion was completed with a GentleMACS Dissociator (Miltenyi Biotec). Lamina propria cells were further purified on a 35% percoll gradient with room temperature centrifugation for 20 min at 2000 RPM with no brake. Cells were washed with PBS, blocked with 2.4G2 (BioXCell, 10 μg/ml), and then stained with LIVE/DEAD Fixable Near-IR (Invitrogen) and anti-mouse antibodies purchased from BioLegend: CD4 (GK1.5), TCRβ chain (H57-597), IL-17A (TC11-18H10.1), CD90.1 (OX-7); or eBioscience: Foxp3 (FJK-16S), IFNγ (XMG1.2). Cells for effector cytokine staining were stimulated at 37° in RPMI with 10% fetal bovine serum (R10) with ionomycin (750 ng/ml), phorbol 12-myristate 13-acetate (50 ng/mL), and GolgiPlug (BD Biosciences). Intracellular staining was performed after cell permeabilization using the Foxp3/Transcription Factor Fixation/Permeabilization Kit (eBioscience). All cells were fixed in 2% paraformaldehyde and acquired with an LSR II cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreeStar).

IL-10 receptor neutralization

Following the end of the ELS-inductive period, 4-week-old mice were injected intraperitoneally with 100 µg anti-IL-10R (BE0050, BioXCell) or vehicle (PBS) (21-040-CV, Corning) every 5 days for 15 days. Injections were administered between 2:00 – 4:00 pm. Mice were euthanized on day 20 or 30 after the initial anti-IL-10R injection.

Assessment of colitis

Representative sections of the proximal, middle, and distal colon were fixed in 2% buffered formalin, paraffin embedded, and 5 µm sections were cut and stained with hematoxylin and eosin (H&E). Histological scoring was performed, using a previously established scheme[25], by a veterinary pathologist who was blinded to the experimental groups. Representative images were collected using Nikon Eclipse Ci microscope and analyzed with NIS-Elements software.

Measurement of CORT, ACTH, and TNF

Blood was collected either by retro-orbital bleeding from isoflurane anesthetized mice or via cardiac puncture at necropsy, all between 8:00 am and 1:00 pm. Plasma was separated by centrifugation at 3,630 x g for 10 minutes at 4°C and stored at -80°C until use. Colonic tissue was rinsed and voided of fecal contents, cut into 1 cm pieces, and incubated at 37°C and 5% CO_2 in 500µl RPMI with 10% FCS (R10) in a 48-well plate. After 48 hours, contents of each well were transferred to a 1.5 ml tube and centrifuged at 20,000 g for 8 minutes and the colon culture supernatant was collected and stored at -80°C until use. Corticosterone levels were detected using the DetectX Corticosterone Enzyme Immunoassay Kit (K014-H5, Arbor Assays). Plasma ACTH levels were detected using the

ACTH Enzyme Immunoassay Kit (EIA-ACTH-5, RayBiotech). TNF levels were detected using the Mouse TNF-alpha DuoSet ELISA kit (DY410-05, R&D Systems). Total protein was determined via the Bicinchoninic acid (BCA) protein assay from colon culture supernatant samples[26]. Colon culture corticosterone levels were normalized to total protein.

Real-time polymerase chain reaction (RT-PCR)

Proximal colonic tissues were homogenized using the Omni tissue homogenizer (Omni International, TH115). Total RNA was extracted from whole colonic tissue or from enriched epithelial cells using TRI Reagent (Zymo Research, R2050-1-200). For colon crypt-enriched epithelial cells, total RNA was extracted using the E.Z.N.A Total RNA Kit 1 (Omega Bio-Tek, R6834-02). Reverse transcription PCR was performed using a C1000 Touch Thermal Cycler (Bio-Rad) with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368813). Real time quantitative PCR was performed on a QuantStudio 3 system (Thermo Fisher Scientific) with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, 1725274). Gene expression was normalized to TATA box binding protein mRNA. Primer sequences can be seen in supplementary data (Table S1).

Isolation and stimulation of colonic crypt cells

Mesenteric fat was removed from the colon prior to flushing luminal contents with PBS. The proximal colonic tissue was cut into 0.2 cm pieces and washed three times via serological pipetting with 10 ml of cold DPBS (MT21031CM, Corning). Tissue pieces were settled by gravity and the supernatant was vacuum aspirated. To release crypt epithelial cells, tissue was digested 3 times for 20 min at 37°C with rotation in collagenase type 1 (2mg/m1; 17-100-017, Corning). The collagenase was neutralized with DPBS

containing 0.1% bovine serum albumin (BSA; BP1600-100, Fisher) and the supernatant containing released epithelial cells was filtered through a 70 µm strainer. The filtered epithelial cell fraction collected after the third digestion step was centrifuged at 290 g for 5 min at 8°C and 1000 cells were suspended in Matrigel (CLS356235, Corning) and plated in a 48-well plate. Cells were cultured with media consisting of 50% L-WRN conditioned media[71], 50% advanced Dulbecco's modified Eagle's medium (DMEM/F-12; D6421, Sigma); supplemented with 20% fetal bovine serum (SH30109.03, Cytiva), 50 µg/ml gentamicin (G1397, Sigma), 10 µM SB 431542, 10 µM Y-27632, 2.5 µg/ml fungizone, 100 units/ml penicillin and 0.1 mg/ml streptomycin (16-141-0, 12-541-0, MT30003CF, MT30002CI, Thermo Fisher Scientific). Some wells were stimulated with 10 ng/ml TNF (T7539, Sigma). Culture supernatants were collected after 48 hours.

Statistical analysis

Statistical significance was calculated by unpaired Student's *t* test, Mann-Whitney U test, one-way ANOVA, two-way ANOVA, or Brown-Forsythe test as appropriate, using Prism software (GraphPad). All $p \leq 0.05$ are considered significant and indicated as such in the text.

RESULTS

ELS in mice disrupts systemic and colonic glucocorticoid production

For our initial studies, we used the maternal separation with early weaning (MSEW) model of ELS, designed to mimic maternal neglect during the suckling period[24]. In this model, newborn pups are separated from the moms daily during the resting period (**Figure 1A**). On day of life (DOL) 2-5, pups were separated for 4 hours daily and then from DOL 6-16 for 8 hours daily. Mice were then weaned on DOL 17, 4 days before their normal rear (NR) counterparts - who were never separated from the moms - were weaned on DOL 21. These experiments were also conducted using our previously developed IL-10 reporter (10BiT) mice[27] to allow simultaneous analysis of IL-10-producing cell subsets known to be critical for the establishment and maintenance of intestinal immune homeostasis. It has been reported that various models of stress including MSEW can result in increased intestinal permeability[28]. In our hands, on DOL 28, we saw no difference in basal permeability between NR and MSEW mice (**Figure 1B**). However, relative to normally reared mice, MSEW mice had significantly lower levels of circulating (CORT) but surprisingly, no difference in ACTH (**Figure 1, C-D**). Significantly lower CORT was also detected in supernatants collected after colon tissue from MSEW mice were cultured *ex vivo* without any additional stimulation (**Figure 1E**). These data argue that ELS can directly impair GC synthesis by both adrenal glands and colonic stem cells independent of any effects on corticosterone-inducing ACTH.

Transient decrease in IL-10-producing T-cells, followed by increased IFNγ$^+$ T-cells in mesenteric lymph nodes of MSEW mice

We assessed live CD4$^+$ T cells of the mesenteric lymph nodes (MLNs) at 4 and 8 weeks of age in NR and MSEW mice to evaluate the impact of ELS on mucosal T cell-dependent immune regulation. There was no difference in the frequency or numbers of Foxp3$^+$CD4$^+$ T cells between NR and MSEW mice at either 4 or 8 weeks of age, indicating that MSEW does not impact basal accumulation of MLN Foxp3$^+$ T regulatory (Treg) cells (**Figure 1, F-G**). Because we utilized the 10BiT mouse line, we were also able to assess IL-10-producing cells based on surface expression of Thy1.1. Interestingly, we found lower frequencies and numbers of IL-10-producing CD4$^+$ T cells at 4 weeks of age in MLNs of MSEW mice relative to NR mice. This difference was no longer present at 8 weeks of age (**Figure 1, F, H**). Conversely, despite similar frequencies and numbers of interferon gamma (IFNγ)-producing CD4 T cells at 4 weeks of age, we observed significantly greater frequencies and numbers of this population in the MLNs of 8-week-old MSEW mice relative to NR counterparts (**Figure 1, I-J**). Collectively these results indicate that MSEW causes delayed differentiation and/or accumulation of mucosal IL-10-producing cells which can enable the emergence of pro-inflammatory Th1 effector cells, thereby altering the mucosal immune cell balance.

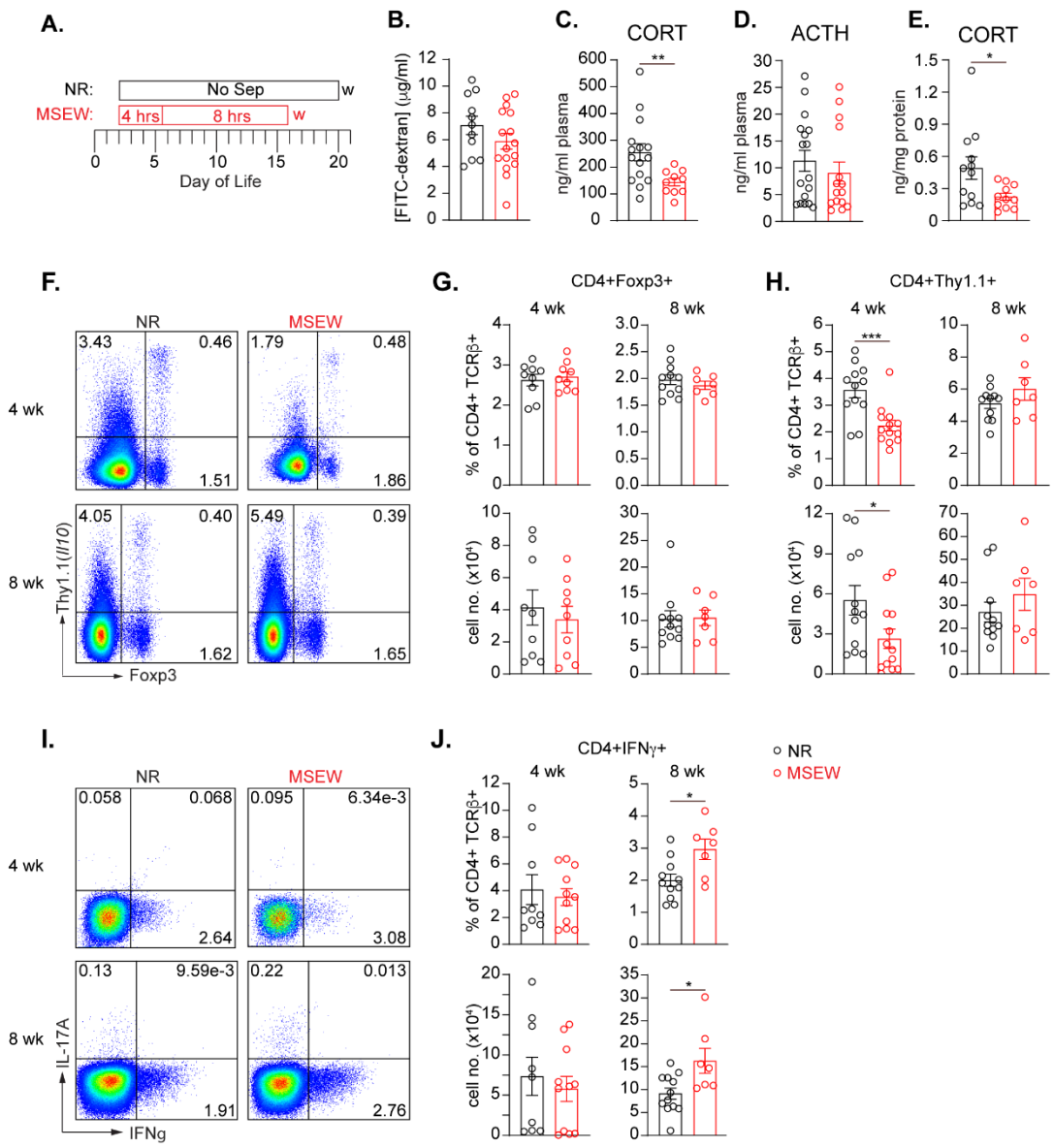
A.
NR:
No Sep
w
MSEW:
4 hrs
8 hrs
w
0
5
10
15
20
Day of Life
B.
[FITC-dextran] (μg/ml)
C.
CORT
ng/ml plasma
**
D.
ACTH
ng/ml plasma
E.
CORT
ng/mg protein
*
F.
NR
MSEW
4 wk
8 wk
3.43
0.46
1.51
1.79
0.48
1.86
4.05
0.40
1.62
5.49
0.39
1.65
Thy1.1 (Il10)
Foxp3
G.
CD4+Foxp3+
4 wk
8 wk
% of CD4+ TCRβ+
cell no. (x10⁴)
H.
CD4+Thy1.1+
4 wk
8 wk
***
*
% of CD4+ TCRβ+
cell no. (x10⁴)
I.
NR
MSEW
4 wk
8 wk
0.058
0.068
2.64
0.095
6.34e-3
3.08
0.13
9.59e-3
1.91
0.22
0.013
2.76
IL-17A
IFNg
J.
CD4+IFNγ+
4 wk
8 wk
*
*
% of CD4+ TCRβ+
cell no. (x10⁴)
NR
MSEW

Figure 1. ELS in mice leads to reduced levels of corticosterone and skewed expression of IL-10 and IFNγ by gut-associated CD4 T cells.

(**A**) Experimental design for maternal separation with early weaning (MSEW). (**B**) Concentration of FITC-dextran in plasma of 4-week-old mice 4 hours after oral gavage and normalized to plasma from mice that did not receive FITC-dextran. Graphs (C-E) showing plasma concentrations of (**C**) CORT and (**D**) (ACTH) in 4-week-old NR and MSEW mice as measured by ELISA ($n \geq 10$ mice/grp). (**E**) CORT concentration in supernatants from 48-hour colon cultures of 4-week-old NR and MSEW mice ($n = 11$ mice/grp). (**F**) Representative flow cytometry plots showing Foxp3 and Thy1.1 expression by live CD4 T cells in the MLNs of NR and MSEW mice at 4- (upper), and 8 (lower) weeks of age. Graphs summarizing frequencies (upper) and numbers (lower) of live $CD4^+$ $Foxp3^+$ (**G**) and live $CD4^+$ $Thy1.1^+$ (**H**) determined as in E. (**I**) Representative flow cytometry plots showing IFNγ and IL-17A expression by live CD4 T cells in the MLNs of NR and MSEW mice at 4 (upper), and 8 (lower) weeks of age. (**I**) Frequency (upper) and numbers (lower) of live $IFN\gamma^+CD4^+$ T cells determined as in H. Error bars represent mean ± SEM. Asterisks denote significance according to Mann-Whitney U test (B-D) or Student's T-test (E-J) at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Data are compiled from 2-3 independent experiments.

MSEW predisposes to sustained intestinal inflammation following a colitogenic insult

To study the effect of ELS on IBD susceptibility and severity, we employed a murine model of colitis predicated on transient disruption of IL10 receptor (IL-10R) signaling. In this model, antibody blockade of the IL-10R results in mild-moderate colonic inflammation that resolves spontaneously following cessation of antibody treatment. Four-week-old NR and MSEW mice received intraperitoneal injections of anti-IL-10R antibody or vehicle (PBS) every 5 days for 15 days (**Figure 2A**). Overall, MSEW mice gained weight more rapidly over the course of the experiment than did NR mice (**Figure 2B**). Mice were euthanized 5 and 15 days after the final α-IL-10R injection, that is, days 20 and 30 respectively after the initiation of IL-10R blockade. As expected, by day 20, there were clear signs of inflammation and epithelial damage that contributed to an overall colitis score that was not different between NR and MSEW mice (**Figure 2, C-D**, upper panels). However, by day 30, and in contrast to significant resolution of colitis in NR mice, MSEW tissue still displayed visible signs of inflammation, epithelial damage and an overall colitis that was virtually unchanged from day 20 (**Figure 2, C-D**, lower panels). Flow cytometric analysis of IFNγ-, IL-17A-, and Foxp3-expressing colonic CD4 T cells revealed that all these subsets were elevated in response to anti-IL-10R treatment but there were no significant differences in frequency or number between NR and MSEW animals (**Figure S1**). We also did not detect significant differences in transcript levels of pro-inflammatory *Il6*, *Il1b*, or *Nos2* (**Figure S2**). However, as early as day 20, there were already significant differences in *Tnf* transcript in colonic tissue from MSEW relative to NR animals and this difference persisted through day 30 (**Figure 2E**). As confirmation, we detected significantly higher TNF protein levels following *ex vivo* culture of colon tissue from

colitic MSEW mice relative to colitic NR mice (**Figure 2F**). Much of this transcript difference appeared to be attributable to non-epithelial cells since we observed small but significant differences in *Tnf* transcript expression among gut tissues crudely denuded of intestinal epithelial cells (IEC) but not among enriched IEC (**Figure S3**). To determine whether CD4 T cells are responsible for the TNF difference, we performed intracellular staining for TNF in live lamina propria CD4 T cells. Unfortunately, we also did not detect significant differences in the frequencies or numbers of CD4+TNF+ cells, neither were there consistent differences in mean fluorescence intensity of TNF (Figure S3, B and C). Altogether, these results support a role for ELS in enhancing individual susceptibility to chronic gut inflammation characterized by sustained production of TNF, potentially by multiple cell subsets.

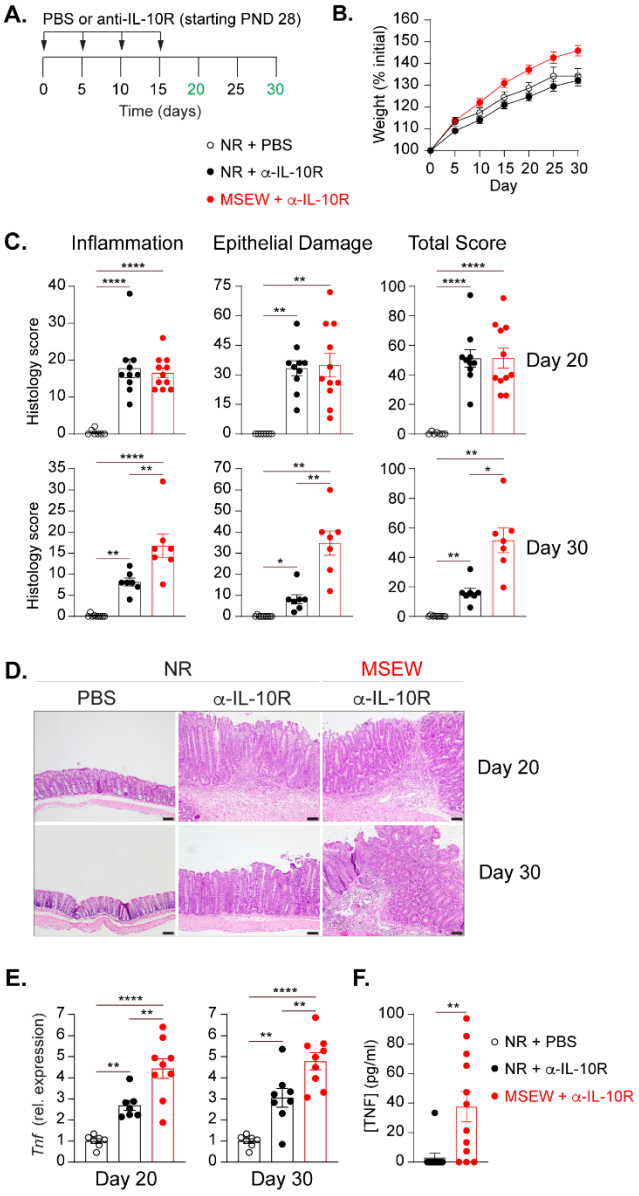
A.
PBS or anti-IL-10R (starting PND 28)
0 5 10 15 20 25 30
Time (days)
NR + PBS
NR + α-IL-10R
MSEW + α-IL-10R
B.
Weight (% initial)
Day
C.
Inflammation
Epithelial Damage
Total Score
Histology score
Day 20
Day 30
D.
NR
MSEW
PBS
α-IL-10R
α-IL-10R
Day 20
Day 30
E.
Tnf (rel. expression)
Day 20
Day 30
F.
[TNF] (pg/ml)
NR + PBS
NR + α-IL-10R
MSEW + α-IL-10R

Figure 2. Blockade of the IL-10 receptor subsequent to ELS results in sustained colitis characterized by elevated *Tnf*.

(**A**) Experimental design for colitis induction. (**B**) Weight change as a percentage of initial (PND 28) weight over the course of the experiment. (**C**) Graphs showing scores for inflammation, epithelial damage, and total colitis of the proximal colon in PBS- or α-IL-10R-treated NR and MSEW male mice at day 20 and day 30. ($n \geq 7$). (**D**) Representative hematoxylin and eosin (H&E) stained colonic tissue sections from PBS- and α-IL-10R-treated NR and MSEW mice. Scale bars = 100μm. (**E**) Relative *Tnf* transcript levels on Day 20 (left), and Day 30 (right) in the proximal colons of NR and MSEW mice treated or not as indicated ($n \geq 7$). All transcripts were initially normalized to TATA box binding protein (*Tbp*). (**F**) TNF concentration in supernatants from 48-hr colon cultures of α-IL-10R-treated NR and MSEW mice on Day 20 ($n \geq 7$). Error bars represent mean ± SEM. Asterisks denote significance from one-way ANOVA (C, E) followed by Tukey's multiple comparisons test or unpaired T-test with Welch's correction (F) at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

ELS induced by early postnatal glucocorticoid exposure recapitulates the effects of MSEW

The foregoing results suggest a link between ELS-induced reductions in systemic and local GC synthesis. Previous studies associate HPA axis dysfunction with an initial spike in the circulating levels of stress-related hormones followed by a persistent decrease in basal corticosterone release[29,30]. A recent study using maternal feeding of the synthetic GC dexamethasone (Dex) during the postnatal period as a model of ELS corroborates the theory that excessive GCs in the early postnatal phase can lead to impaired output of corticosterone later in life[31]. Therefore, we asked whether early postnatal introduction of glucocorticoids can mimic the effects of MSEW. Timed pregnant females were randomly assigned to control or ELS groups and fed Dex or vehicle (Veh) in drinking water from the day after delivery (postnatal day 1, PND 1) until PND 14. All cages were returned to regular drinking water immediately thereafter and progeny were weaned as normally done on PND 21 (**Figure 3A**). As with the MSEW model, we also observed no effect of Dex on basal colonic permeability at 4 weeks of age (**Figure 3B**). However, there were significantly lower levels of plasma CORT, but not ACTH, in Dex mice relative to Veh controls (**Figure 3, C-D**). We then examined whether Dex also predisposed to chronic colitis using the IL-10R blockade model where Dex or Veh mice were injected intraperitoneally with PBS or anti-IL-10R antibody every 5 days for 15 days (**Figure 3E**). Similar to MSEW mice, Dex mice also gained weight more rapidly during the experimental period (Figure 3F). Also, like MSEW mice, we observed significant differences in the extent of colon epithelial damage and overall disease severity in Dex mice compared to Veh mice (**Figure 3G**). Specifically, colons from Veh mice showed an inflammatory infiltrate limited to the lamina propria and mild crypt epithelial hyperplasia whereas colons from Dex mice had

significantly increased inflammatory infiltrate and epithelial erosion (**Figure 3H**). Furthermore, like the MSEW model, we did not observe significant differences in transcripts of *Il6* or *Il1b*, their respective receptors, or *Nos2* (**Figure S3**). However, Dex-treated mice displayed elevated *Tnf* mRNA 30 days post initiation of anti-IL-10R injections (**Figure 3I**). Importantly, this was accompanied by increased transcripts of *Tnfrsf1a* and *Tnfrsf1b* (which encode TNF receptors 1 and 2, respectively) in crypt-enriched colonic epithelial cells (**Figure 3J**). Collectively, these data demonstrate that increased GCs in the early postnatal period impairs GC output and can contribute to enhanced severity and chronicity of any inflammation that subsequently develops in the colon. Moreover, this chronic inflammation is accompanied by increased *Tnf* transcription and seemingly elevated TNF responsiveness by CORT-producing colonic cells.

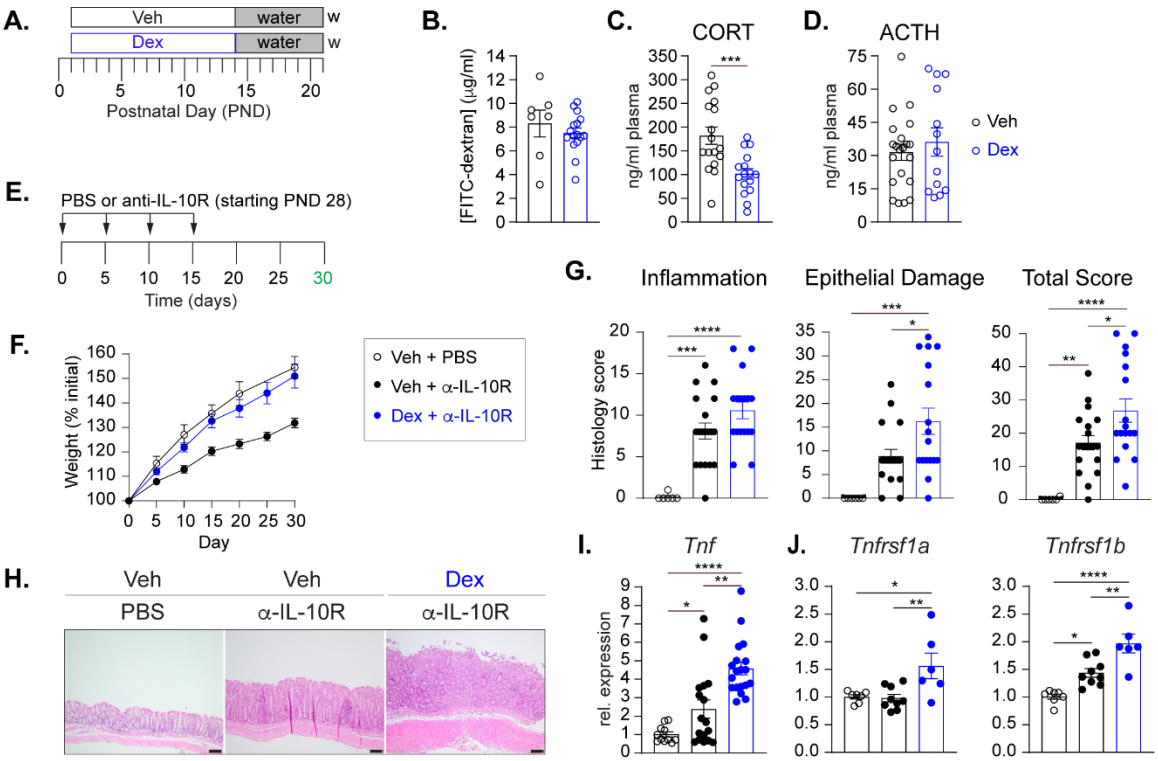

Figure 3. Early postnatal exposure to glucocorticoids recapitulates the effects of MSEW on adolescent mice.

(A) Experimental design for early life exposure (day of life 1 – 14) to dexamethasone (Dex) or the vehicle (Veh, 0.002% DMSO, 0.02% 2-hydroxypropyl-β- cyclodextrin). (**B**) Concentration of FITC-dextran in plasma of 4-week-old mice 4 hours after oral gavage and normalized to plasma from mice that did not receive FITC-dextran. Graphs (C-D) showing plasma concentrations of (**C**) CORT and (**D**) (ACTH) in 4-week-old Veh and Dex mice as measured by ELISA ($n \geq 13$). (**E**) Experimental design for colitis induction, beginning at 4-wks of age, 2-wks post Dex exposure. (**F**) Weight change as a percentage of initial (PND 28) weight over the course of the experiment. (**G**) Graphs showing scores for inflammation, epithelial damage, and total colitis of the proximal colon in α-IL-10R-treated Veh and Dex mice on day 30. ($n \geq 17$). (**H**) Representative H&E-stained colonic tissue sections from PBS- and α -IL-10R-treated Veh and Dex mice Scale bars = 100μm. (**I**) Relative *Tnf* mRNA levels in proximal colons of α-IL-10R-treated Veh and Dex mice normalized to PBS-treated Veh mice ($n \geq 10$ mice/group). (**J**) Relative mRNA levels of *Tnfrsf1a* (left) and *Tnfrsf1b* (right) in crypt-enriched epithelial cells from the colons of α-IL-10R-treated Veh and Dex mice, normalized to PBS-treated Veh mice ($n \geq 6$). All transcripts were initially normalized to *Tbp*. Error bars represent mean ± SEM. Asterisk denotes significant difference from Mann-Whitney U test (C), or one-way ANOVA followed by Tukey's multiple comparisons test (G, I, J) at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****). Data are compiled from 3 individual experiments.

ELS leads to reduced colonic CORT levels at steady state without suppression of *Nr5a2*

The effects of ELS extend well into adulthood as has been demonstrated in both humans and animal models. Our results demonstrating the effects of 2 different models of ELS on systemic and colonic GC production shortly after cessation of stress exposure (4 weeks of age) raised the possibility of a mechanistic link between the impaired GC synthesis and the colitis observed. Furthermore, given similar outcomes observed in mice subjected to MSEW or maternally transmitted Dex, we conducted side-by-side examination of the effects of both regimens on colonic GC synthesis in the absence of any additional perturbations. In 8-week-old adult mice previously subjected to either MSEW or Dex, colonic corticosterone levels were consistently and significantly lower than that of their control counterparts (**Figure 4A**). However, we found no differences between mice from either ELS cohort or their control counterparts in the colonic transcripts of the GC receptor *Nr3c1* (**Figure 4B**) suggesting that any downstream effects of reduced GC under these 'homeostatic' conditions may not be due to reduced potential for ligand-receptor interactions. We also found no differences in colonic expression of *Nr5a2* (which encodes Lrh-1), suggesting the differences in colonic corticosterone at steady state are also not regulated at the level of Lrh-1 expression (**Figure 4C**).

ELS and TNF additively suppress colonic CORT output

We then sought to determine the potential effects of ELS on GC synthesis and responsiveness under inflammatory conditions by comparing colonic tissues of colitic MSEW and Dex mice normalized to unstressed, non-colitic animals. We found that overall, inflammation caused modest reductions in *Nr3c1* in both stressed and unstressed mice

(**Figure 4D**). However, this decrease was even more pronounced and significantly different in colitic Dex mice relative to Veh-exposed counterparts (**Figure 4D**, lower). A similar trend was seen in the transcript levels of *Nr5a2*, where inflammation promoted lower expression in both models with a clearly significant difference observed between Dex and Veh mice (**Figure 4E**). When we examined the expression of the *Nr5a2* transcriptional target *Cyp11a1* we found that inflammation was associated with a significant up-regulation in transcript levels of C*yp11a1* in colitic but unstressed mice. However, this upregulation was either tempered, or completely inhibited, in colitic MSEW and Dex mice, respectively (**Figure 4F**). These findings suggest that ELS has the potential to modulate intestinal GC synthesis pathway, especially in mice subjected to Dex during the suckling period. This was not surprising given that this approach involves more direct and controlled manipulation of the early GC spike believed to be associated with ELS. We thus focused our remaining experiments on mice exposed to vehicle or Dex. In addition to the sustained deficit in local CORT production at steady state that was found in both ELS models (Figure 4A), we also confirmed that there is diminished intestinal CORT output during active colitis from Dex mice relative to Veh (**Figure 4G**) – providing further support for impaired steroidogenic pathway induction in colitic ELS animals. We next asked whether TNF, the most reproducibly up-regulated inflammatory mediator detected in our experiments, was central to the inflammation-induced downregulation of GC synthesis. Indeed, a role for TNF in suppression of colonic GC synthesis has been described[32]. We isolated colonic crypt cells from healthy Veh and Dex mice and cultured *in vitro* supported by stem cell survival factors in the presence or absence of exogenous TNF. Like the cultures of whole colon tissue (Figure 4A), unstimulated crypt cells from Dex mice spontaneously secreted

lower levels of corticosterone than cells from Veh mice. Addition of TNF inhibited the production of corticosterone in both circumstances, but importantly, there was a more significant reduction in expression of corticosterone production by cells from Dex-exposed mice (**Figure 4H**). Collectively, these data demonstrate that TNF-driven local inflammation potently suppresses colonic GC synthesis and that this effect is further exacerbated by prior exposure to ELS.

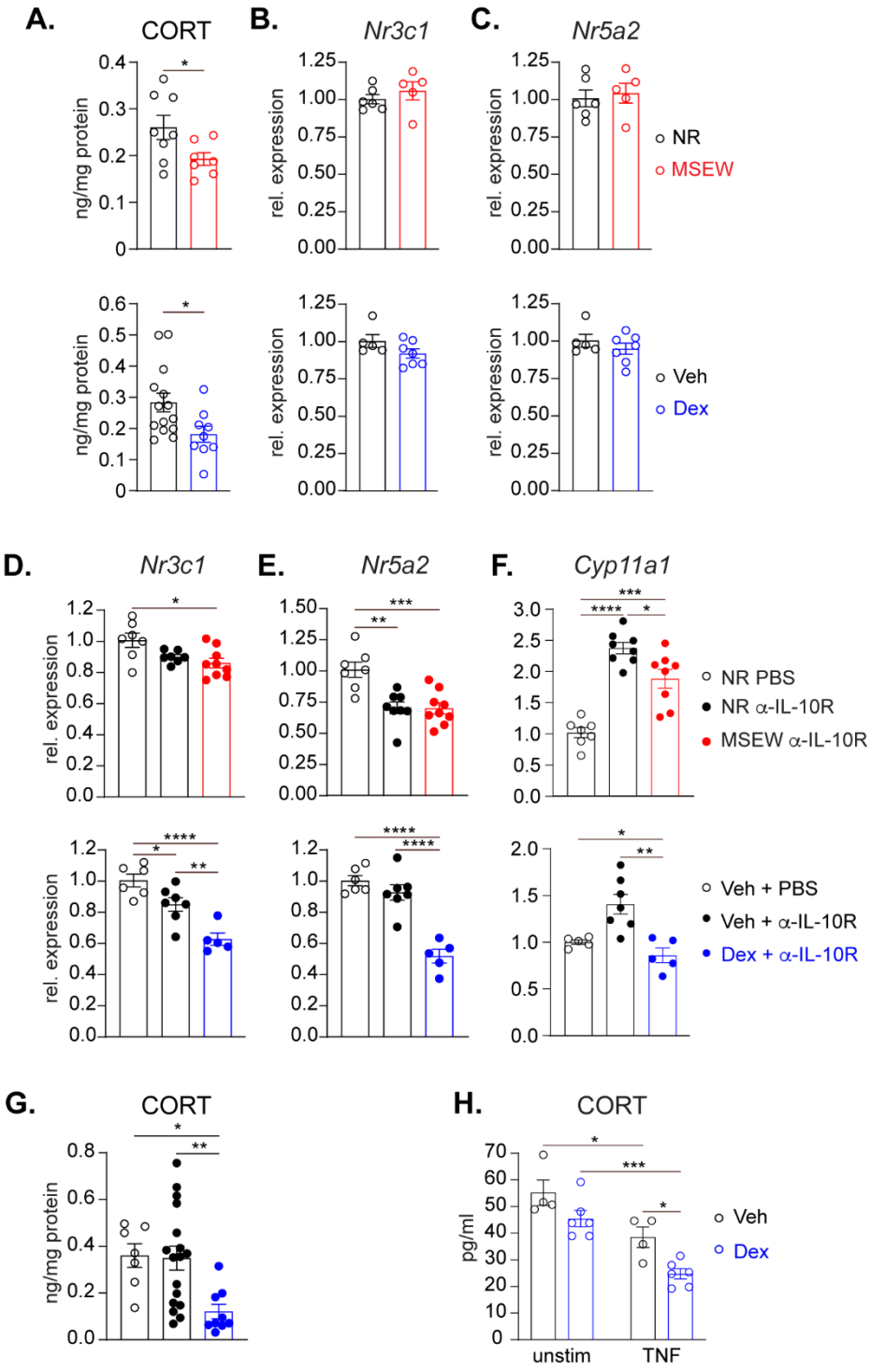
A.
CORT
ng/mg protein
B.
Nr3c1
rel. expression
C.
Nr5a2
rel. expression
NR
MSEW
Veh
Dex
D.
Nr3c1
E.
Nr5a2
F.
Cyp11a1
rel. expression
NR PBS
NR α-IL-10R
MSEW α-IL-10R
Veh + PBS
Veh + α-IL-10R
Dex + α-IL-10R
G.
CORT
ng/mg protein
H.
CORT
pg/ml
unstim
TNF
Veh
Dex

Figure 4. ELS and TNF suppress colonic CORT output.

(**A**) Corticosterone concentration in supernatant after 48-hour cultures of colon from 8-week-old NR and MSEW (upper); and Veh and Dex mice (lower) ($n \geq 7$). (**B**) Relative expression of *Nr3c1* and (**C**) *Nr5a2* in proximal colons of healthy NR versus MSEW (upper) and Veh versus Dex (lower) mice ($n \geq 5$). (**D**-**F**) Relative expression of (D) *Nr3c1*, (E) *Nr5a2*, and (F) *Cyp11a1*, in proximal colons of α-IL-10R-treated NR and MSEW mice (upper), and Veh and Dex mice (lower), normalized to PBS-treated NR and Veh mice, respectively ($n \geq 7$). All transcripts were initially normalized to *Tbp*. (**G**) Corticosterone concentration in supernatant after 48-hour cultures of colon from 8-week-old α-IL-10R-treated Veh and Dex mice at Day 30 ($n \geq 9$). (**H**) Corticosterone concentration as determined by ELISA in supernatant of proximal colonic crypt cells generated from 8-week-old Veh and Dex mice stimulated or not with 10 ng/ml TNF stimulation for 48-hr ($n \geq 4$). Error bars represent mean ± SEM. Asterisks denote significant differences from Student's T-test at (A), one-way (D-G) or two-way (H) ANOVA followed by Tukey's multiple comparisons test at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

DISCUSSION

In this study we have examined the effects of ELS on mediators of intestinal immune regulation and on the chronic phase of colonic inflammation induced secondarily to ELS. As validation of our approach, we showed that whether induced via MSEW or postnatal dexamethasone exposure, ELS resulted in HPA axis dysfunction, evidenced by reduced circulating levels of CORT. In addition, we demonstrate for the first time, that ELS also leads to impaired production of CORT in the colon both in the adolescent period shortly after removal of the stress signal and in adulthood. This reduced local CORT may be central to the increased inflammatory tone of the intestine, which was characterized by early deficits in mucosal IL-10-producing CD4 T cells followed by increased IFNγ-producing CD4 T cells in early adulthood. Although ELS has been reported to alter intestinal barrier integrity and promote low grade inflammation in mice[28,33-36], in non-colitic MSEW and Dex mice, we did not detect differences in basal epithelial permeability or any overt pathology relative to control mice. Manifestation of the effects of ELS on gut inflammation required the introduction of a colitogenic insult for which we employed antibody-mediated blockade of the IL-10 receptor. Thus, our study demonstrates how this environmental trigger can induce and imprint disruptions in normal gut immune homeostasis and can have long term effects on the predisposition to, and perpetuation of, chronic inflammation in susceptible hosts (**Figure 5**).

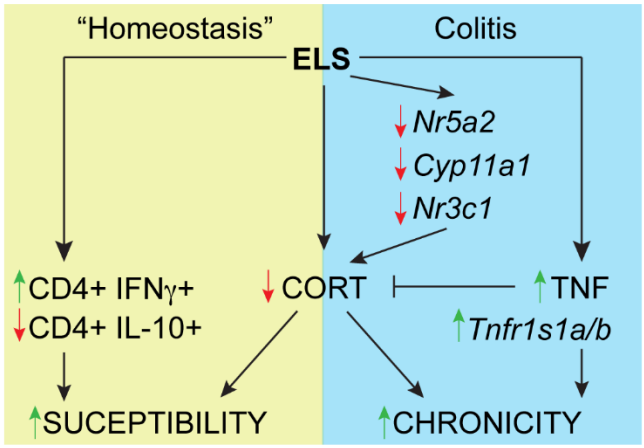

Figure 5. Proposed model of the impact of ELS in health and disease.

ELS results in reduced production of colonic corticosterone (CORT). In otherwise healthy ELS mice under "homeostatic" conditions, ELS also alters the mucosa-associated immune cell balance with an initial deficit in anti-inflammatory IL-10-producing CD4 T cells followed by an increase in IFNγ-producing CD4 T cells. This diminished local CORT and altered mucosal immune milieu enhance the susceptibility of ELS-exposed individuals to inflammation especially in the presence of additional colitogenic insults. During active colitis, local deficits in CORT production and function are further reinforced via reductions in (1) *Nr5a2*, the gene encoding Lrh1 that is responsible for colonic CORT production, (2) *Cyp11a1*, which encodes the first enzyme involved in *de novo* steroidogenesis, and (3) *Nr3c1*, which encodes the global GCR. Conversely, colitis in ELS animals is consistently associated with (1) increased intestinal TNF production, which can further suppress local CORT synthesis, and (2) increased transcription of genes encoding TNF receptors 1 and 2. The combination of increased TNF and reduced CORT contributes to a sustained inflammation in the colons of ELS-exposed mice.

The literature reports contradictory effects of ELS on circulating GC concentrations in both humans and laboratory animals with GCs elevated in some settings[37,38] and decreased in others[39-41]. The change in CORT is often directly related to changes in the levels of circulating ACTH, the primary activator of CORT release by adrenal glands. Despite the reduced CORT, in both models of ELS, we did not detect significant changes in circulating ACTH. Yet, although we did not directly assess adrenal gland CORT secretion, impaired adrenal gland GC output in response to stress is the most likely reason for the reduced plasma CORT levels in our ELS mice. Notably, in the absence of inflammation in the intestine, we also did not detect changes in expression of *Nr5a2*, the gene encoding Lrh-1 that transcriptionally regulates colonic CORT synthesis. Thus, both models of ELS resulted in lower basal levels of adrenal- and intestinal-derived CORT without affecting expression of the established upstream mediators of GC synthesis and/or release.

In contrast to the otherwise steady state, under inflammatory conditions we detected ELS-dependent reductions in expression of *Nr5a2* and *Cyp11a1*, and a corresponding significant decrease in intestinal CORT output from colitic ELS mice relative to colitic unstressed mice. Our findings are consistent with the reported down-regulation of *NR5A2* in the colons of ulcerative colitis patients[42]. Colon tissue from colitic ELS mice also showed lower expression of *Nr3c1*, the gene encoding the GCR. Thus, during inflammation, prior exposure to ELS contributes to reduced CORT output and signaling which collectively, can contribute to the chronicity of colitis via inadequate control of pro-inflammatory mediators. Interestingly, all these transcripts were altered in unstressed mice with colitis, demonstrating effects of inflammation independent of ELS. However,

particularly in colitic Dex mice, each was more substantially altered/downregulated suggesting additive effects of ELS and inflammation. The reason for the discordance in GC receptor transcripts in control versus ELS colons during health and disease is currently unclear and further studies will be required to delineate the potentially distinct mechanisms underlying ELS mediated suppression of CORT synthesis and sensitivity in the colon under the 2 conditions.

Chronic inflammation in mice is characterized by upregulation of numerous pro-inflammatory pathways in both innate and adaptive immune effector cells. Accordingly, we found that independent of ELS, there was elevated Th1 and Th17 cells and increased mRNA of pro-inflammatory cytokines IL-1β and IL-6 in colonic tissue of colitic mice. However, across multiple experiments in 2 different models, *Tnf* was the only factor examined for which we consistently detected significantly higher transcript levels in ELS mice relative to control mice. We were unable to identify a single cell population that is primarily responsible for the elevated Tnf in ELS mice. At a minimum, our data appears to exclude epithelial cells and potentially CD4 T cells as being solely responsible. Thus, we speculate that the elevated TNF may be a net effect of moderate over-production by multiple cell types in the lamina propria. Our results also suggest that expression of the GCR is a major feature of the culpable cell type(s) and the near ubiquitous expression of the GCR further supports the notion that multiple cellular sources of TNF may be involved.

Despite not identifying a primary source of TNF, our results suggest that CORT-producing colonic crypt stem cells are important targets of TNF in colitic ELS mice. Specifically, crypt-enriched colonic epithelial cells harbored increased transcripts of the genes encoding TNF receptors 1 and 2 - but not those encoding IL-1 or IL-6 receptors –

and produced less CORT in the presence of TNF. This led us to explore the potential intersection of TNF and local colonic CORT in mediating the long-term effects of ELS in colitic mice. Even prior to our study, it was known that this is a complex relationship. For example, in acute colitis in mice, TNF administration can trigger CORT synthesis to reduce disease[43]. Conversely, in chronic colitis, TNF can prevent CORT synthesis via suppression of *Nr5a2* and TNF blockade can restore intestinal CORT synthesis to limit disease activity[44]. Interestingly, in the same study, lower amounts of TNF over a shorter duration induced expression of intestinal steroidogenic genes, suggestive of a dose-dependent relationship[44]. In our study, we found that the ELS-induced deficit in colonic CORT existed under 'homeostatic' conditions when TNF levels were minimal or undetectable and persisted well into the chronic phase of colitis which was characterized by constantly elevated TNF. Furthermore, *in vitro* stimulation of colonic crypt cells with high dose TNF - used to mimic severe chronic inflammation - resulted in significantly reduced CORT output, especially in cells from Dex-exposed mice. This inhibition of CORT synthesis by TNF is likely self-perpetuating. Both the GCR and TNF can be expressed by multiple intestinal cell types during inflammation, meaning that inadequate CORT signaling to any or all the major TNF-producing subsets can result in failure to regulate TNF production and potentially sustain the inflammatory reaction. Collectively then, our study supports a model wherein ELS predisposes to prolonged colitis via insufficient local production of CORT via direct targeting of crypt stem cells by TNF coupled with reduced GCR availability enabling additional TNF production and signaling.

The exact molecular pathway whereby ELS targets and imprints colonic crypt cells also remains to be elucidated. Studies in humans and rodents have uncovered ELS-induced

epigenetic alterations in multiple stress-response genes across various regions of the brain[45,46]. These changes have been found to impact GC output and alter the functional signaling capability of the GCR, but the relationship, if any, to local colonic CORT is unclear. Early life is also a critical developmental window for the gut microbiota, which is also impacted by ELS[47-50]. For example, we recently observed differences in microbiota composition between NR and MSEW mice characterized by lower abundance of a number of short chain fatty acid (SCFA)-producing taxa[50], which may explain the delayed emergence of regulatory IL-10-producing CD4 T cells observed in 4-week-old MSEW mice. The second model employed in this study involved maternal ingestion of Dex which could also potentially alter both the microbiota and the brain physiology of the pups. Interestingly, despite the very different approaches, we observed similar GC-related phenotypes in both models before and after induction of inflammation, suggesting that both regimens caused similar microbial changes or that the net effect of any microbial differences is minimal relative to the effects of reduced colonic CORT synthesis.

In summary, our study demonstrates how exposure to chronic stress during early life can lead to defective gut immune regulation, including inadequate colonic CORT production. Throughout our study, we observed that several albeit statistically significant ELS-dependent phenotypes and outcomes were not particularly dramatic relative to those of the control counterparts. Instead, the overarching effects of ELS appear to be cumulative and synergistic with additional colitogenic perturbations and served to exacerbate the effects of inflammation. Our findings are consistent with the multi-factorial nature of IBD and demonstrate how this specific environmental trigger can contribute to worse disease outcomes in susceptible individuals (**Figure 5**).

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Author Contributions

RQM, RGL, JSP, and CLM conceived and designed research. CLM supervised research. RQM performed all experiments with assistance from BJK, MSJ, PAM, JSH, AND CEK. JBF performed colitis scoring. GL helped with interpretation of histological images and generated photomicrographs. RQM AND CLM wrote the manuscript.

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ETHICAL CONSIDERATIONS

Disclosure of Financial Interest

RGL is a current employee of Genentech, a member of the Roche group, and may hold Roche stock or stock options. All other authors declare that they have no competing financial interests.

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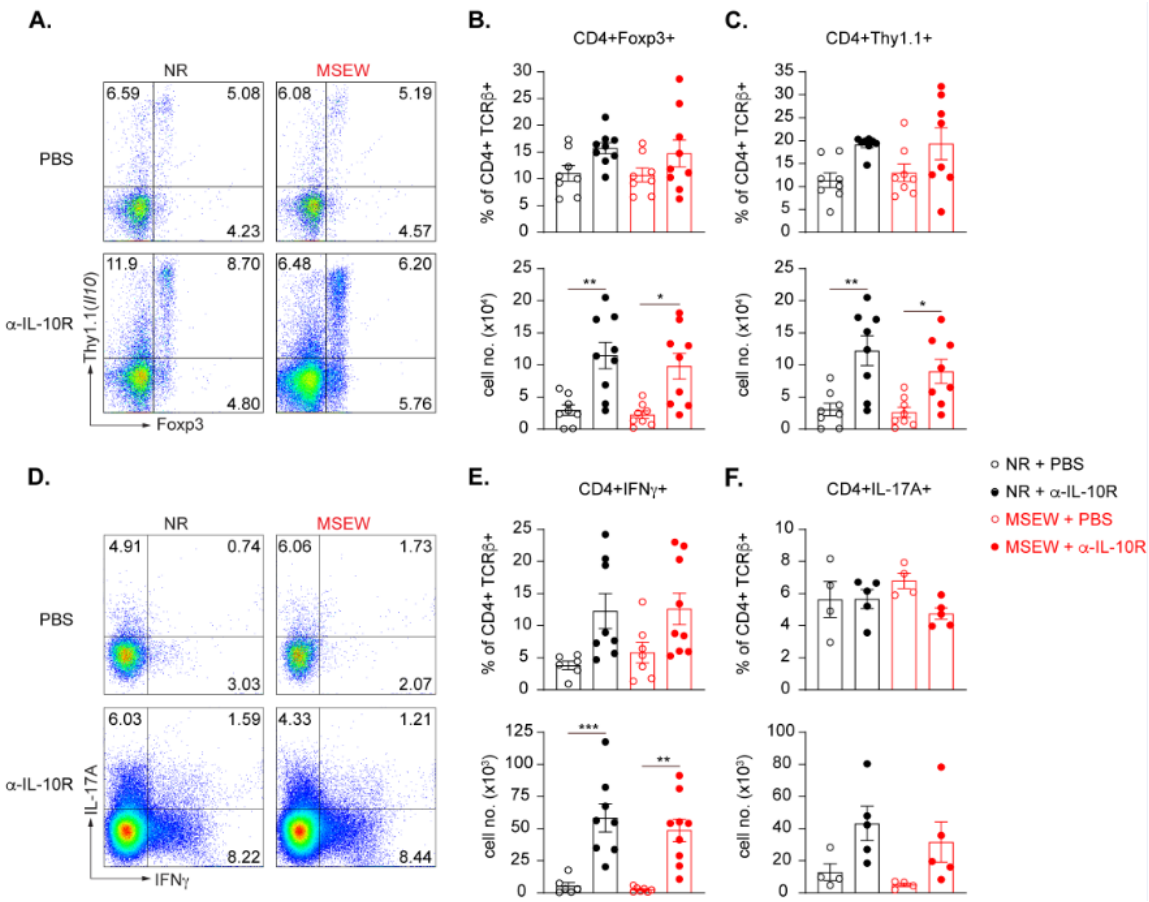

Figure S1. Chronic colitis induces increased colonic Foxp3-, IL-10-, and IFNγ-, and IL-17A-expressing CD4 T cell populations independent of ELS.

(**A**) Representative flow cytometry plots showing Foxp3 and Thy1.1 expression by live CD4 T cells in the colons of PBS vehicle control and anti-IL-10R treated NR and MSEW mice at 8 weeks of age, 15 days after the last anti-IL-10R injection. (**B-C**) Graphs summarizing frequencies (upper) and numbers (lower) of live $CD4^+$ $Foxp3^+$ (**B**) and live $CD4^+$ $Thy1.1^+$ ((**C**) ($n \geq 8$)). (**D**) Representative flow cytometry plots showing IFNγ and IL-17A expression by live CD4 T cells in the colons of PBS vehicle control and anti-IL-10R treated NR and MSEW mice at 8 weeks of age, 15 days after the last anti-IL-10R injection. (**E-F**) Frequency (upper) and numbers (lower) of live $IFN\gamma^+CD4^+$ T cells (**E**) and live $IL\text{-}17A^+CD4^+$ T cells ((**F**) ($n \geq 4$)). Error bars represent mean ± SEM. Asterisks denote significance from two-way ANOVA (B, C, E, F) followed by Tukey's multiple comparisons test at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Data are compiled from 2-3 independent experiments.

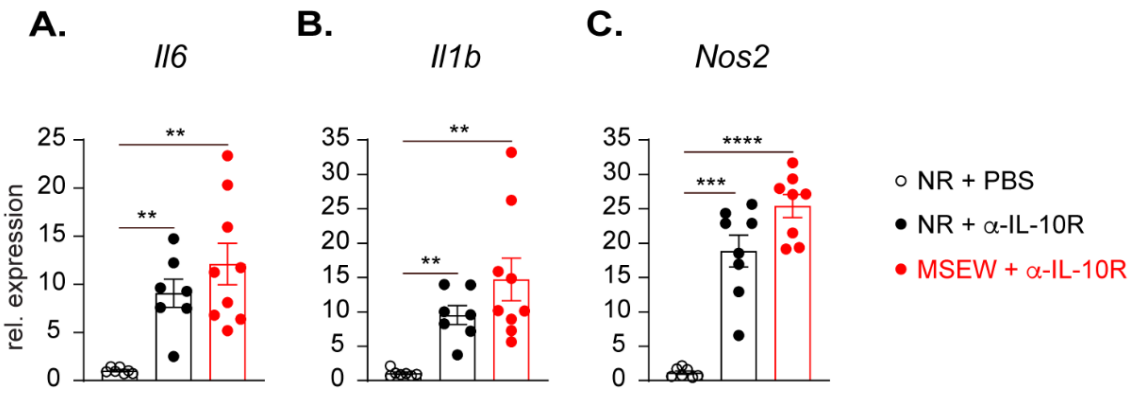

Figure S2. MSEW does not lead differential induction of Il6, Il1b, or Tnf when mice are subjected to colitis.

Relative mRNA levels of (**A**) *Il6*, (**B**) *Il1b*, and (**C**) *Nos2* in proximal colons of α-IL-10R-treated NR and MSEW mice normalized to PBS-treated NR mice ($n \geq 7$). All transcripts were initially normalized to *Tbp*. Error bars represent mean ± SEM. Asterisks denote significant differences from Brown-Forsythe and Welch ANOVA followed by Dunnett's T3 multiple comparisons test (A-C) -at $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

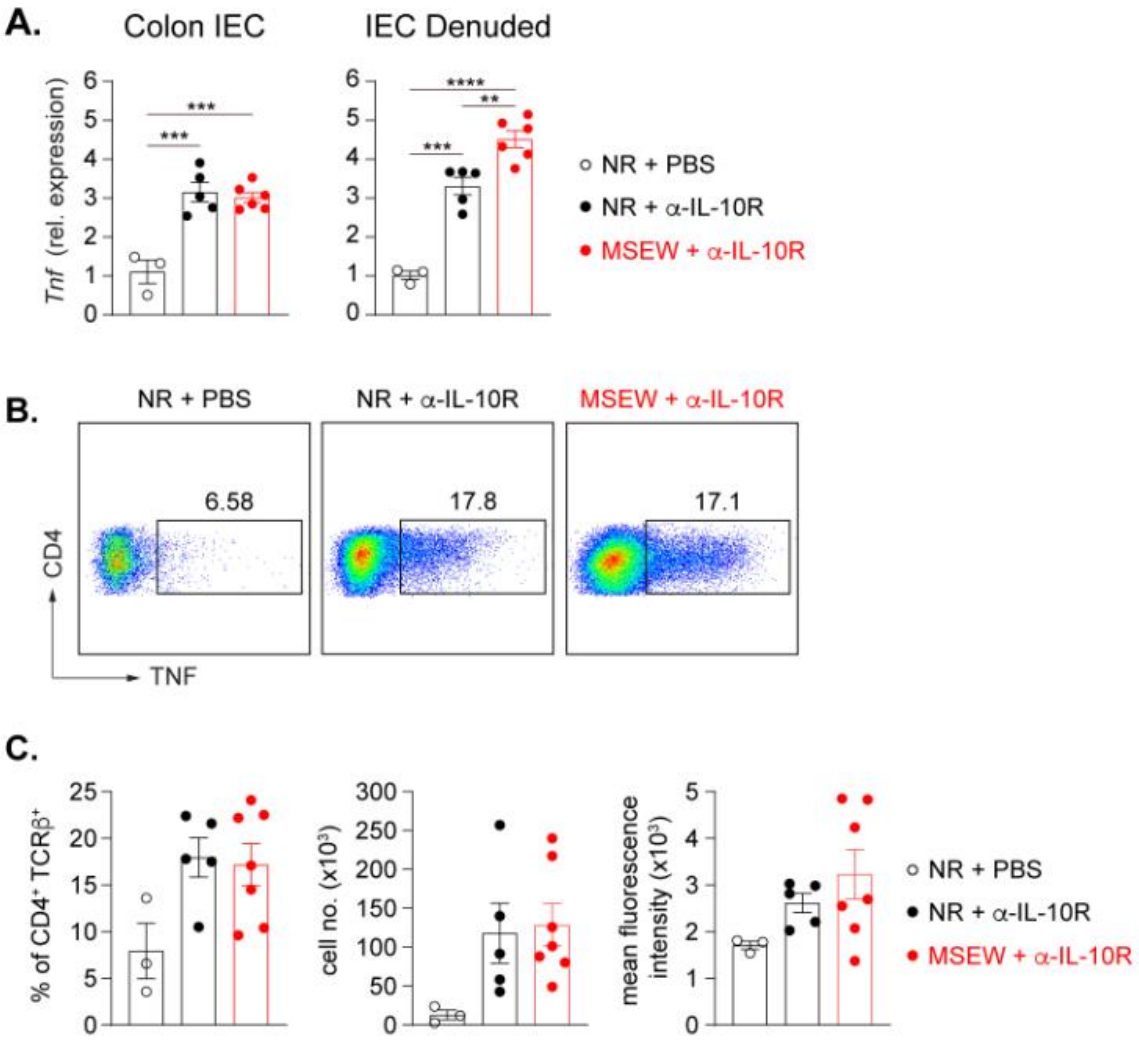

Figure S3. Elevated induction of Tnf by non-epithelial cells in colitic MSEW mice relative to NR counterparts.

(**A**) Relative *Tnf* mRNA levels from epithelial cells (left) or epithelial-depleted colonic tissue (right) of α-IL-10R treated NR and MSEW male mice at Day 20, normalized to PBS-treated NR mice ($n \geq 3$). All transcripts were initially normalized to TATA box binding protein (*Tbp*). (**B**) Representative flow cytometry plots showing TNF expression by live CD4 T cells in the colons of PBS treated NR and anti-IL-10R treated NR and MSEW mice on day 20. (**C**) Frequency (left), number (middle), and mean fluorescent intensity (MFI, right) of live $TNF^{+}CD4^{+}$ T cells ($n \geq 3$). Asterisks denote significance from one-way ANOVA (A) followed by Tukey's multiple comparisons test at $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

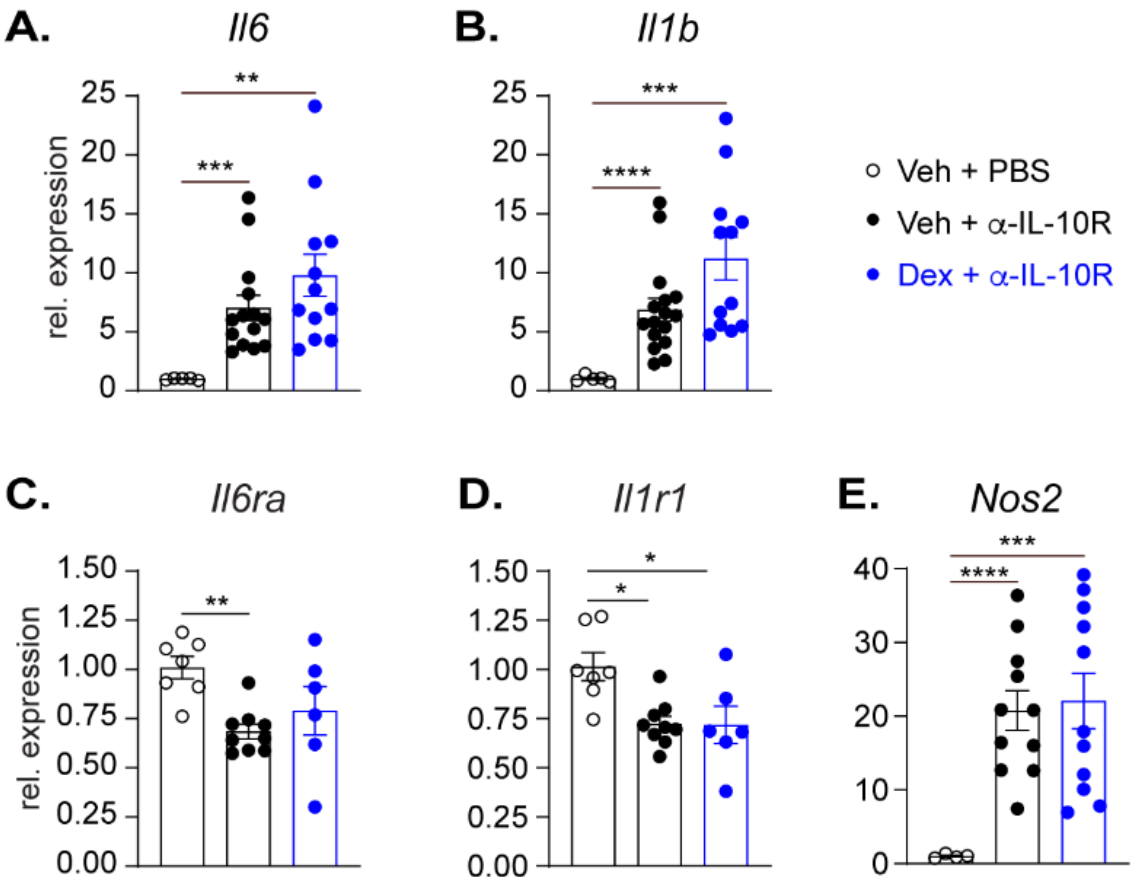

Figure S4. Dex does not significantly impact *Il6* or *Il1b* induction or responsiveness when mice are subjected to colitis.

Relative mRNA levels of (**A**) *Il6*, (**B**) *Il1b*, (**C**) *Il6ra*, (**D**) *Il1r1*, and (**E**) *Nos2* in proximal colon tissue (A, B, E) or crypt-enriched epithelial cells from the colons (C, D) of α-IL-10R-treated Veh and Dex mice, normalized to PBS-treated Veh mice ($n \geq 4$). All transcripts were initially normalized to *Tbp*. Error bars represent mean ± SEM. Asterisks denote significant differences from Brown-Forsythe and Welch ANOVA followed by Dunnett's T3 multiple comparisons test (A, B, E) or one-way ANOVA followed by Tukey's multiple comparisons test (C, D) at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

TABLE S1: Primers used in qRT-PCR experiments.

Gene	Forward	Reverse
Cyp11a1	5'-CTGCCTGGGATGTGATTTTC-3'	5'-ACACCAGGGTACTGGCTGAA-3'
Il1b	5'-GCAACTGTTCCTGAACTCAACT-3'	5'-ATCTTTTGGGGTCCGTCAACT-3'
Il1r1	5'-AGCCAGTTTTATCGCTATCCG-3'	5'-ATAAAGCCCCCGATGAGGTA-3'
Il6	5'-CACTTCACAAGTCGGAGGCT-3'	5'-TTCTGCAAGTGCATCATCGT-3'
Il6ra	5'-AAGACTCTGCCAACCACGAG-3'	5'-CAAGCTTCCTCCAGCTACCA-3'
Nos2	5'-TGCATGGACCAGTATAAGGCAAGC-3'	5'-GCTTCTGGTCGATGTCATGAGCAA-3'
Nr3c1	5'-GTCAAGGTTTCTGCGTCTTC-3'	5'-AAGGTGCT TTGGTCTGTGG-3'
Nr5a2	5'-TTGAGTGGGCCAGGAGTAGT-3'	5'-ACGCGACTTCTGTGTGTGAG-3'
Tbp	5'-ACCGTGAATCTTGGCTGTAAAC-3'	5'-GCAGCAAATCGCTTGGGATTA-3'
Tnf	5'-CCCTCACACTCAGATCATCTTCT-3'	5'-GCTACGACGTGGGCTACAG-3'
Tnfrsf1a	5'-CCGGGAGAAGAGGGATAGCTT-3'	5'-TCGGACAGTCACTCACCAAGT-3'
Tnfrsf1b	5'-CAGGAAGGCTCAGATGTGCT-3'	5'-CATGCTTGCCTCACAGTCC-3'

COLONIC ENDOTHELIN RECEPTOR TYPE A IS REGULATED BY THE MICROBIOTA AND HELPS TO CONTROL COLONIC PERMEABILITY

by

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Format adapted for dissertation

ABSTRACT

The role of the endothelin (ET) system in regulating blood pressure, renal, and cardiovascular health and disease has been extensively studied, but there is a gap in our knowledge about the potential role of the ET system in mediating intestinal homeostasis. Although previous publications have observed protection from chemically induced colitis with the use of endothelin receptor antagonists, little has been done to assess the mechanism by which these drugs are providing this protection. Additionally, the microbiota has been identified as a crucial component for vascular homeostasis, but the mechanism whereby the microbiota regulates vascular physiology remains unclear. Thus, we hypothesized that the microbiota may regulate colonic expression of the ET system and that ET receptor antagonism protects against colitis by maintaining intestinal barrier integrity. Direct comparison between germ-free (GF) and specific pathogen-free (SPF) mice revealed colonic gene expression differences for ET receptors and peptides. The presence of a microbiota resulted in downregulation of colonic transcripts for ET receptors and upregulation of ET peptides. Further, microbial conventionalization of adult gnotobiotic mice restored colonic gene expression of ET receptors back to levels observed in SPF mice. Administration of Atrasentan – an endothelin receptor type A (ET_A) antagonist – for 6 days prior to colitis induction via dextran sulfate sodium (DSS) administration, was sufficient to prevent DSS-induced pathology, reflected in histological scoring. Prophylactic ET_A antagonism prevented DSS-induced disruptions of both the epithelial and endothelial barriers in the colon. Further, gene expression data from colonic

barrier cells suggests that ET_A antagonism maintained barrier integrity by preventing DSS-induced alterations in junctional proteins (*Cdh5*, *Cldn1*). Lastly, upregulation of *Plvap* in colonic barrier cells with prophylactic Atrasentan treatment suggests that ET_A antagonism may even have enhanced endothelial barrier integrity. Collectively, our study demonstrated that the microbiota regulates colonic expression of the ET system and implicates ET_A-mediated signaling in colitis pathogenesis via colonic barrier disruption.

INTRODUCTION

The endothelium is a single layer of cells making up the inner layer of all blood vessels and the lymphatic system. The endothelium is a dynamic organ that must actively sense and respond to environmental cues in order to physiologically regulate vascular tone, vascular smooth muscle migration, and cellular adhesion[1]. The endothelin (ET) system includes 3 structurally similar 21 amino acid peptides (ET-1, ET-2, and ET-3) signaling through one of two G-protein coupled receptors – ET receptor type A or type B (ET_A, ET_B). Alterations of the ET system are involved in endothelial dysfunction which phenotypically manifests as increased vascular tone, elevated expression of cellular adhesion molecules, and compromised barrier function – leading to edema and increased leukocyte extravasation[2]. Thus, endothelial dysfunction has been linked to various chronic inflammatory conditions[3], including inflammatory bowel disease (IBD)[4].

Inflammatory bowel disease is a complex disease of the gastrointestinal tract with multifactorial etiology involving genetic susceptibility, environmental factors, the microbiota, and aberrant immune responses. Abnormal intestinal vascular morphology and function has been detected in affected tissue of IBD patients, with the extent of vascular abnormalities correlating with the severity of disease[5-7]. The endothelin system has previously been implicated in IBD pathogenesis. ET-1 is elevated in the plasma of Crohn's disease (CD) and ulcerative colitis (UC) patients[8] and affected intestinal tissue biopsies have exhibited increased endothelin reactivity[9-11]. Additionally, previous rodent studies have found that the use of endothelin receptor antagonists prevents intestinal damage in

mice administered trinitrobenzene sulfonic acid (TNBS) or dextran sulfate sodium (DSS) [12-14]. One study utilized intravital microscopy to demonstrate that the use of a non-selective endothelin receptor antagonist, Bosentan, concurrently with DSS was able to reduce intestinal leukocyte adhesion back to levels observed in untreated water controls[15]. Despite the protective effects of endothelin receptor antagonism in colitis studies, the mechanism of action remains unclear.

In addition to the involvement in cellular adhesion and leukocyte diapedesis, the ET system has also been linked to endothelial hyperpermeability in various disease states. It was recently demonstrated that ET-1 signaling through ET_A mediates endothelial hyperpermeability in systemic capillary leak syndrome[16] and promotes glucose-induced increases in endothelial permeability in diabetes [17]. Involvement of both ET receptors have been implicated in various diseases or injuries of the brain that compromise blood brain barrier (BBB) and thus endothelial barrier integrity[18-21]. Recently, the concept of the gut-vascular barrier (GVB) was proposed, inspired by the restricted flow of molecules and ions characteristic of the BBB. Rescigno et al. demonstrated the existence and importance of the GVB, in addition to identifying structural similarities between the BBB and GVB[22], i.e. enteroglial cells and pericytes in close contact with intestinal vascular endothelial cells comprising the GVB-unit and reminiscent of the BBB-unit involving close contact of astroglial cells, pericytes, and brain vascular endothelial cells. The apparent similarities between the vascular barriers of the brain and gut suggests that ET_A-mediated vascular hyperpermeability observed in the brain, may also occur in the gut and contribute to the perpetuation of inflammation with IBD.

Dysregulated microbiota is a key feature of IBD, though it remains unclear whether this precedes IBD onset or is a result of the disease. The microbiota and the human gut have coevolved for millions of years, resulting in an important regulatory role of the microbiota in human health[23]. Regarding the vasculature, previous studies have found that the microbiota is necessary for maturation of the intestinal vascular network. For example, germ-free (GF) mice exhibit immature or arrested capillary network formation and microbial conventionalization of adult GF mice results in rapid angiogenesis and maturation of intestinal blood vessels[24,25]. Expanding on this, Joe et al. have found that the microbiota is essential for vascular physiology[26]; GF animals are hypotensive and blood pressure can be restored to physiological levels by microbial reconstitution[27]. Taken together, it seems plausible that the microbiota may impact expression of the endothelin system within the colon.

In this study we first determined whether the microbiota contributes to colonic expression of the endothelin system. Secondly, we investigated how ET_A antagonism is protective against colitic intestinal injury. Comparisons between GF and coventional mice revealed that the presence of a microbiota results in downregulation of colonic transcripts for ET receptors and upregulation of colonic transcripts for ET peptides. Additionally, we show that microbial reconstitution of adult GF mice restored colonic ET receptor transcript expression back to levels observed in specific pathogen-free mice. We validated earlier findings that ET_A antagonism prevented DSS-induced colitis and demonstrate that ET_A antagonism yields this protective effect by maintaining colonic barrier integrity, at the level of both the epithelium and endothelium. Gene expression analysis within the colon suggests that this maintenance of barrier integrity involves both endothelial-specific and

non-specific endothelial and epithelial junctional proteins. Collectively, our results demonstrate that the microbiota regulates colonic expression of the ET system and ET_A-mediated signaling contributes to IBD pathogenesis via colonic barrier disruption.

METHODS

Mice

All mice used in this study were on the C57BL/6 genetic background, originally acquired from Jackson Laboratories (Bar Harbor, ME). Unless otherwise indicated in the figure legend, male mice were used in DSS colitis experiments due to the well-documented observation that female mice are more resistant to the development of DSS-induced colitis [28,29]. Germ free (GF) and altered Schaedler flora (ASF) mice were obtained from the UAB Gnotobiotic Mouse Core (GMC). GF, ASF, and conventionalized GF mice were maintained at the GMC for the duration of the experiment. Specific pathogen-free (SPF) mice were bred and maintained in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines.

Real-time polymerase chain reaction (RT-PCR)

Either whole or distal colonic tissues were homogenized using the Omni tissue homogenizer (Omni International, TH115). Total RNA was extracted from colonic tissue or from enriched colonic barrier cells using TRI Reagent (Zymo Research, R2050-1-200). Reverse transcription PCR was performed using a C1000 Touch Thermal Cycler (Bio-Rad) with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368813). Real time quantitative PCR was performed on a QuantStudio 3 system (Thermo Fisher Scientific) with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad,

1725274). Gene expression was normalized to TATA box binding protein mRNA. Primer sequences:

Gene	Forward	Reverse
Cdh5	5'-TTGGAATCAAATGCACATCG-3'	5'-TCTTGCCAGCAAACTCTCCT-3'
Cldn1	5'-ACTGTGGATGTCCTGCGTTT-3'	5'-TGCCAATTACCATCAAGGCT-3'
Ednra	5'-CTGAAAACAATTTTTGAATTTCTTGC-3'	5'-TACCAAGATGTGAAGGACTGGTGG-3'
Ednrb	5'-CGGACTACAAAGGAAAGCCC-3'	5'- CCACCAATCTTTGGCTGTC-3'
Edn1	5'-GCACCGGAGCTGAGAATGG-3'	5'-GTGGCAGAAGTAGACACACTC-3'
End2	5'-CTCCTGGCTTGACAAGGAATG-3'	5'-GCTGTCTGTCCCGCAGTGTT-3'
Ocln	5'-CATTTATGATGAACAGCCCCC-3'	5'-GGCCATTGGACTGTCAACTC-3'
Plvap	5'-GAGATGGAGCGCATCAACG-3'	5'-CTTCAGCTGTTCCTGGCACT-3'
Tbp	5'-ACCGTGAATCTTGGCTGTAAAC-3'	5'-GCAGCAAATCGCTTGGGATTA-3'
Tjp1	5'-GTTCAGAGCCCTCCGATCAT-3'	5'-GCTCTTCCTCTCTGCTCCG-3'

Microbial conventionalization of gnotobiotic mice

Contents from the lumen of the colons of 8-wk SPF mice were collected into sterile phosphate-buffered saline (PBS). Lumenal content was centrifuged for 1 min at 250 g at 4°C and the supernatant was collected for microbial conventionalization of GF mice. GF mice were gavaged both orally and rectally with microbial content suspended in PBS. Conventionalized mice were maintained in isocages within the Gnotobiotic Mouse Core at UAB for 4 weeks until the experimental endpoint.

Quantification of bacterial load

Fecal pellets were collected and weighed prior to extraction using the Quick-DNA Fecal/soil Microbe Miniprep kit (Zymo, D6010). Fecal DNA samples were amplified via qPCR using the SSO Advanced Universal Sybr Mix (BioRad, 1725274) and the microbial 16s specific primer set: 515F (5' – GTGYCAGCMGCCGCGGTAA) and 805R (5' – GACTACHVGGGTATCTAATCC). Data was analyzed using the Thermo Fisher Connect Design and Analysis qPCR software. Standard curves were generated creating serial dilutions of a plasmid containing the 16s gene from *Lachnospiraceae A4*. The copies of 16s were normalized to fecal weight using the following equation: $\frac{copies}{g} = \frac{(16s\frac{copies}{uL}) \times dilution\ factor \times elution\ volume\ (uL)}{fecal\ weight\ (g)}$.

Endothelin receptor antagonism

Mice received either the selective ET_A antagonist, Atrasentan (ABT-627; AbbVie Laboratories, North Chicago, IL) or dual endothelin receptor antagonism via Atrasentan and A-192621 (ET_B antagonist; PepTech Corp., Bedford, MA) ad libitum in the drinking

water at a dose of 10 mg/kg/day for a total of 6 days. Water was changed after 3 days with fresh antagonist.

Induction of colitis

Dextran sulfate sodium (DSS; MW 36–50 kDa, MP Biomedicals, Santa Ana, CA) was administered at a concentration of 1-2% (w/v) dissolved in drinking water; DSS concentration had to be adjusted due to differential potency between batches/lots. For **Figure 3** and **Supplemental Figure 2**, mice were kept on 1% DSS water for 6 days, followed by 4 days of regular drinking water before the experimental endpoint. For the assessment of intestinal epithelial permeability in **Figure 4A**, mice were exposed to 3 days of 2% DSS prior to the FITC-dextran assay. For the assessment of intestinal endothelial permeability in **Figure 4B**, mice were exposed to 4 days of 2% DSS prior to the Miles assay. For the assessment of colonic epithelial and endothelial junctional protein transcript expression in **Figure 4C-G**, mice were exposed to 2% DSS for 2 days. From the start of DSS administration, body weight was measured daily through the duration of the experiment.

Assessment of colitis

Representative sections of the proximal, middle, and distal colon were fixed in 2% buffered formalin, paraffin embedded, and 5 μm sections were cut and stained with hematoxylin and eosin (H&E). Histological scoring was performed, using a previously established scheme [30], by a veterinary pathologist who was blinded to the experimental groups. Representative images were collected using Nikon Eclipse Ci microscope and analyzed

with NIS-Elements software. Additional parameters that were collected for the assessment of DSS colitis include[31,32]: colon length, colon weight, and spleen weight.

Assessment of intestinal epithelial permeability

Mice were fasted for six hours prior to administration of 4-kDa FITC-dextran (Sigma-Aldrich, 400 mg/kg body weight). Blood was obtained via the retro-orbital plexus into heparinized capillary tubes, 4 hr post-gavage. Plasma was collected via centrifugation at 10,000 g for 10 minutes at room temperature. Plasma was diluted 1:4 in sterile deionized (DI) water in a 96-well plate. Fluorescence was detected by a Synergy HT spectrophotometer (Biotek; excitation: 485 nm, emission: 528 nm). A standard curve of FITC-dextran dissolved in sterile DI water at final concentrations ranging from 0 - 10,000 ng/ml was used to calculate FITC-dextran concentrations of the samples. To eliminate background signal, fluorescent emission signals in the plasma of mice that did not receive FITC-dextran were averaged and subtracted from emission signals of mice treated with 4-kDa FITC-dextran.

Assessment of intestinal endothelial permeability

We used the previously published, Miles assay to evaluate *in vivo* vascular permeability[33]. Briefly, 0.1% Evans Blue solution (100 µL; Allied Chemical) was injected into the circulation via the retro-orbital plexus. Thirty minutes after the injection, mice were sacrificed by cervical dislocation. Colons were collected, voided of luminal content, and weighed. Colons were submerged in 250 µl of formamide (Fisher Scientific, BP1160-500) and incubated at 55°C for 24 h. Samples were centrifuged at 10000 *g* for 30 minutes. The

supernatants were collected, and absorbance was detected by a Synergy HT spectrophotometer (Biotek) at 610 nm.

Collection of colonic barrier cells

Colons were stripped of mesenteric fat, were flushed with sterile PBS, and cut open longitudinally. Tissue was approximately sectioned into 1-cm pieces and incubated 3 separate times for 20 minutes at 37°C with rotation in 154 μg/L L-dithioerythritol and 2 μM ethylenediaminetetraacetic acid (EDTA) in Hank's balanced salt solution (HBSS) to remove the mucus and barrier cells. After each incubation, supernatants containing barrier cells were collected into a sterile container and kept on ice. After the third and final incubation, all 3 fractions were combined and centrifuged at 500 *g* for 8 minutes at 4°C. Colonic cell fractions were washed, re-pelleted, suspended in RNA*later* solution (Invitrogen, AM7021), and stored at -80°C for RNA extraction at a later date.

Statistical Analysis

Statistical significance was calculated by unpaired Student *t* test, one-way ANOVA, two-way ANOVA, or Kruskal-Wallis test followed by multiple comparisons as appropriate, using Prism software (GraphPad). All $p \leq .05$ values are considered significant and indicated as such in the text.

RESULTS

The microbiota regulates endothelin system expression in the colon

In order to investigate if the microbiota regulates expression of endothelin system components in the colon, we performed quantitative RT-PCR on colon tissue from germ-free (GF) and specific pathogen free (SPF) mice at 3- and 8-wks of age. The 8-wk samples clearly indicate a role for microbial regulation of endothelin system components within the colon; where colonic transcripts for both *Ednra/b* are significantly upregulated in the absences of a microbiota (**Figure 1A, B**) and transcripts for both *Edn1/2* are significantly downregulated in the absence of a microbiota (**Figure 1C, D**). Differences in transcript expression between 3-wk and 8-wk samples indicate that there is another factor, in addition to the microbiota, that also appears to regulate colonic expression of endothelin system components. While 3-wk old SPF animals exhibit colonic transcript levels for *Ednra/b* similar to that of their 8-wk counterparts, 3-wk GF animals display significantly decreased colonic *Ednra/b* relative to their 8-wk counterparts – indicating that there is another regulatory force at play (**Figure 1A, B**). Inversely for the colonic transcript expression of the peptides, 3-wk SPF animals display significantly less *Edn1* expression relative to their 8-wk counterparts while 3-wk GF animals exhibit similar *Edn1* expression relative to their 8-wk counterparts (**Figure 1C**). Interestingly, 3-wk SPF animals demonstrate significantly less *Edn2* levels relative to their 8-wk counterparts and to the 3-wk GF colons (**Figure 1D**). Whereas the 3-wk GF animals exhibit significantly more colonic *Edn2* relative to their 8-wk counterparts (**Figure 1D**). All together, the data indicates that there is indeed a

regulatory component of the microbiota on colonic expression of the endothelin system, but also that there is another regulatory mechanism at play that appears to work temporally.

Additionally, we also assessed colonic transcript expression of endothelin system components between 8-wk SPF, GF and altered Schaedler flora (ASF) mice. ASF is a model consortium of 8 known commensal microbes that represent a defined murine microbiota. ASF-colonized mice exhibit normal GI function and a mature immune system, making it an ideal mouse model for the study of host-microbiome interactions within a defined consortium of colonized microbes[34]. While 8-wk SPF and GF colons revealed results that were reflective of those observed in **Figure 1** 8-wk SPF vs GF samples, ASF colons resembled GF transcript levels for *Ednra/b* and *Edn1/2* (**Sup Figure 1A-D**), despite the fact that ASF mice exhibited a similar fecal bacterial load to SPF mice (**Sup Figure 1E**). These results suggest that a complex microbiota is necessary for the regulatory expression of colonic endothelin system genes or that none of the 8 specific microbes making up the ASF community can specifically alter colonic expression of *Ednra/b* or *Edn1/2*.

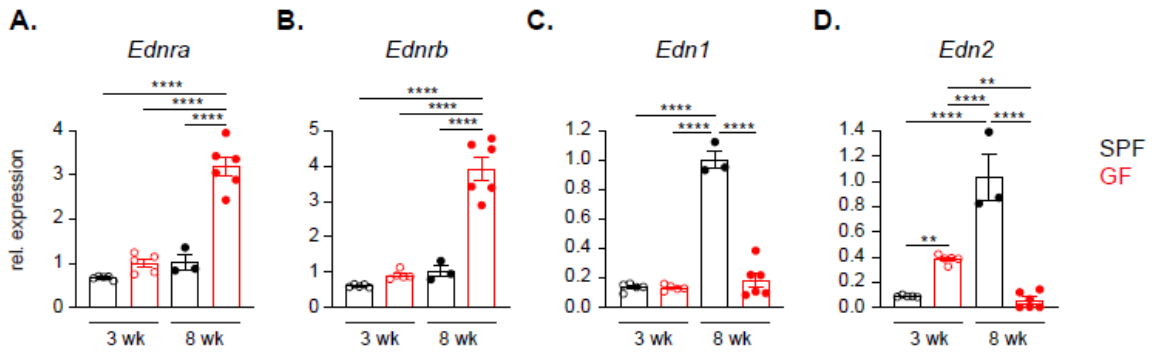

Figure 1. The microbiota regulates endothelin system expression in the colon.

Fold change in *Ednra* (**A**), *Ednrb* (**B**), *Edn1* (**C**), *Edn2* (**D**) transcript expression from colons of SPF and GF mice at 3- and 8-wks of age. All transcripts were initially normalized to TATA box binding protein (*Tbp*). Error bars represent mean ± SEM. Asterisks denote significance from two-way ANOVA followed by Tukey's multiple comparisons test at $p < 0.01$ (**) and $p < 0.0001$ (****). Data are from one experiment.

Microbial conventionalization of GF animals restores colonic endothelin receptor transcript expression but not endothelin peptide transcripts

The differences in colonic transcript expression of endothelin system components between GF and SPF mice led us to investigate if microbiota conventionalization of adult GF mice would restore colonic *Ednra/b* and *Edn1/2* expression to SPF levels. Luminal bacteria from 8-week-old SPF mice were transplanted into age-matched GF recipients. We verified GF status prior to microbial reconstitution (**Figure 2E, left**) and confirmed successful colonization (**Figure 2E, right**) via quantification of bacterial 16S ribosomal DNA. Four weeks after microbial reconstitution, colons were collected for assessment of endothelin system transcript expression. Colonization of adult GF mice resulted in the restoration of colonic *Ednra* and *Ednrb* expression back to levels observed in SPF mice (**Figure 2A, B**). However, conventionalization had no effect on colonic transcripts for of *Edn1* or *Edn2*. (**Figure 2C, D**). Taken together, these results indicate that the microbiota has a role in the regulation of colonic endothelin receptors.

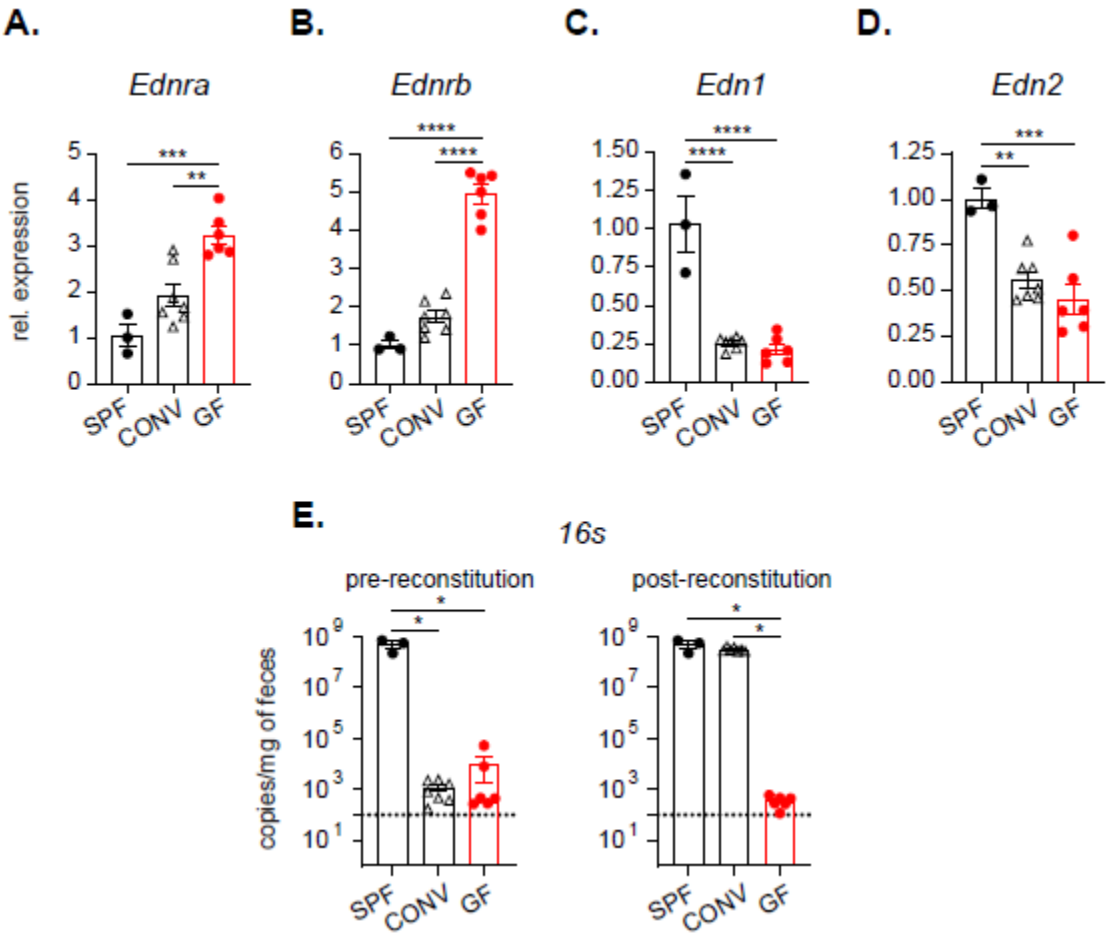

Figure 2. Microbial conventionalization of gnotobiotic mice restores colonic *Ednra/b* expression but not *Edn1/2*.

Fold change in *Ednra* (**A**), *Ednrb* (**B**), *Edn1* (**C**), *Edn2* (**D**) transcript expression from epithelial-denuded colonic tissue of SPF, conventionalized (CONV), and GF mice relative to SPF ($n \geq 3$). All transcripts were initially normalized to *Tbp*. (**E**) Fecal 16S rRNA copy number pre-conventionalization (left) and post-conventionalization (right). Error bars represent mean ± SEM. Asterisks denote significance from one-way ANOVA followed by Tukey's multiple comparisons test (A-D) or Kruskal-Wallis followed by Dunn's multiple comparisons test (E) at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Data are from one experiment.

Prophylactic endothelin receptor antagonism is protective against DSS-induced colitis

A possible role for endothelial involvement in mediating IBD pathogenesis has previously been described[3,4]. Additionally, DSS-induced colitis resulted in upregulation of colonic *Ednra/b* and downregulation of colonic *Edn1/2* (**Sup Figure 2A-D**). This, along with the regulatory component of the microbiota on transcript expression of the endothelin receptors in the colon (**Figure 1, 2**), led us to investigate how prophylactic endothelin receptor antagonism may impact DSS-induced colitis. Initial experiments included experimental groups that were pre-treated for 6 days with either the selective ET_A antagonist, Atrasentan (ABT-627) or dual endothelin receptor antagonism via Atrasentan and A-192621, an ET_B antagonist. A schematic of the treatment conditions is featured in **Figure 3A** (below the body weight tracking graph). Daily body weight assessment revealed expected weight loss in DSS-treated animals (**Figure 3A**). Prophylactic endothelin receptor antagonism largely prevented DSS-induced weight loss – with more protection observed in animals that were pre-treated with the dual ET receptor antagonist (**Figure 3A**).

In addition to weight loss, DSS-treated animals also exhibited expected colon shortening (**Figure 3B**) and increased colon and spleen weight (**Figure 3C, D**). Animals that were treated with ET receptor antagonists prior to DSS exposure displayed values similar to water controls for colon length and spleen and colon weight (**Figure 3, B-D**) – indicating protection from DSS-induced colitis. Despite the increased protection from weight loss observed in **Figure 3A** with dual ET receptor antagonism versus selective ET_A antagonism, there were no differences between pre-treated groups for colon length or spleen and colon weight (**Figure 3, B-D**). DSS mice displayed significant histological signs

of pathology – evidenced by inflammation and epithelial damage (**Figure 3E, F**). Prophylactic ET receptor antagonism largely prevented DSS-induced signs of colonic pathology (**Figure 3E, F**). Despite the fact that dual ET receptor antagonism appeared to prevent weight loss more so than selective ET_A antagonism (**Figure 3A**), selective ET_A antagonism prior to DSS exposure more effectively prevented histological signs of colitis, with this difference being most apparent in the inflammation score (**Figure 3E, left**). There were no major differences in disease outcomes between selective ET_A or dual ET receptor antagonism prior to DSS exposure, indicating that ET_A antagonism alone is sufficient to prevent colitis. Altogether, the data implicates ET_A in IBD pathogenesis.

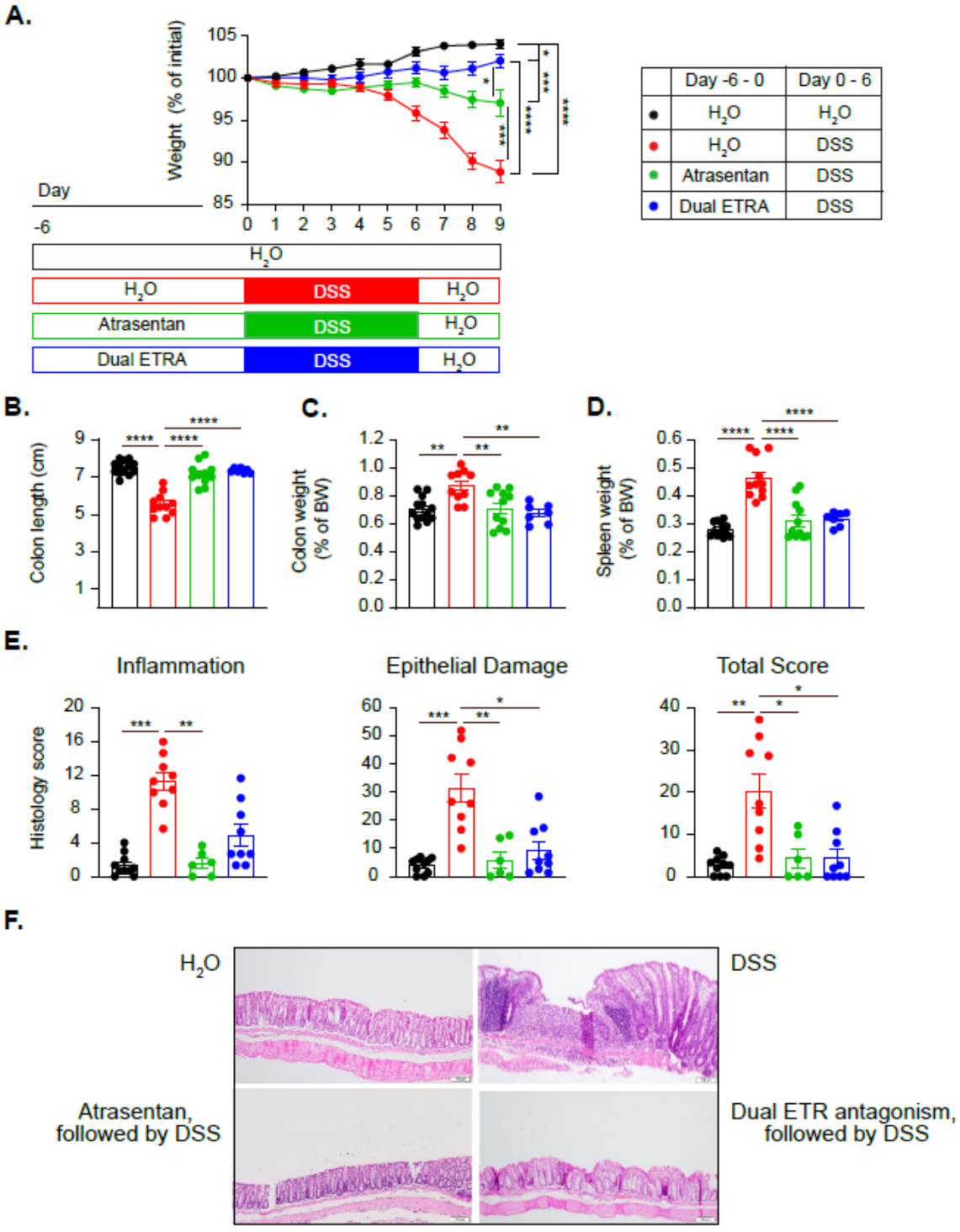
A.
Weight (% of initial)
105
100
95
90
85
0 1 2 3 4 5 6 7 8 9
Day
-6
H2O
H2O
DSS
H2O
Atrasentan
DSS
H2O
Dual ETRA
DSS
H2O
Day -6 - 0
Day 0 - 6
H2O
H2O
H2O
DSS
Atrasentan
DSS
Dual ETRA
DSS
B.
Colon length (cm)
C.
Colon weight (% of BW)
D.
Spleen weight (% of BW)
E.
Inflammation
Epithelial Damage
Total Score
Histology score
F.
H2O
DSS
Atrasentan, followed by DSS
Dual ETR antagonism, followed by DSS

Figure 3. Prophylactic endothelin receptor antagonism is protective against DSS-induced colitis.

(**A**) Percent change in body weight beginning with DSS administration. (**B**) Colon length in centimeters (cm). Colon weight (**C**) and spleen weight (**D**) normalized to body weight at experimental endpoint ($n \geq 7$). (**E**) Quantified inflammation score (left), epithelial damage score (middle), and the sum total histological score (right) ($n \geq 6$). (**F**) Representative hematoxylin and eosin (H&E) stained colonic tissue sections from each group. Scale bars = 100 μm. Error bars represent mean ± SEM. Asterisks denote significance from two-way (A) or one-way (B-D) ANOVA followed by Tukey's multiple comparisons test or from Kruskal-Wallis followed by Dunn's multiple comparison test (F) at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Data are compiled from two individual experiments.

Prophylactic ET_A antagonism prevents DSS-induced disruption of colonic epithelial and endothelial barriers

To confirm the protection from epithelial damage with prophylactic ET receptor antagonism observed in **Figure 3E**, we assessed colonic epithelial permeability via oral administration of FITC-dextran followed by photometric quantification of FITC detected within the plasma 4 hours post-gavage. Mice were pre-treated for 6 days with the selective ET_A antagonist, Atrasentan followed by 3 days of DSS prior to colonic permeability assessment – as previous studies have reported that 3 days of DSS exposure is sufficient to detect altered barrier integrity[35,36]. DSS-treated mice displayed elevated colonic epithelial permeability relative to water controls, whereas ET_A antagonism prior to DSS exposure prevented increases in intestinal epithelial permeability (**Figure 4A**). We also assessed endothelial permeability via the Miles assay, wherein Evans Blue dye is injected into the circulation where it binds strongly with albumin, which is typically retained within the vascular space. Detectable extravasation of dye-labelled albumin indicates protein leakage and thus compromised endothelial barrier integrity[33]. Atrasentan was administered for 6 days prior to 4 days of DSS exposure before endothelial permeability assessment. DSS resulted in elevated colonic endothelial permeability, whereas prophylactic ET_A antagonism prevented colonic endothelial barrier disruption (**Figure 4B**).

This protection of barrier integrity led us to investigate the gene expression of various junctional proteins from enriched colonic barrier cells after acute exposure to DSS (2 days). While the cellular fraction assessed was enriched for epithelial cells, it is likely that endothelial cells were contained within the fraction, as EDTA is a calcium chelator – leading to cadherin inhibition and disruption of cellular adhesion[37]. Additionally, colonic capillaries are situated in very close proximity to the epithelium (1 μm)[38] and EDTA has

been shown to detach/release both endothelial and epithelial cells from underlying tissue[37,39-41]. Thus, we assessed gene expression of junctional proteins that participate in both epithelial and endothelial barrier formation (claudin-1, occludin, and Zonula occludens-1 (ZO-1); encoded by *Cldn1*, *Ocln*, and *Tjp1* respectively). Additionally, we assessed gene expression of junctional proteins that are specific to endothelial cells, such as vascular endothelial-cadherin (VE-cadherin, encoded by *Cdh5*)[42] and plasmalemma vesicle-associated protein (PV-1, encoded by *Plvap*)[43]. Despite the fact that mice were only exposed to 2 days of DSS, we were able to detect significant differences in some of the junctional protein transcripts from colonic barrier cells. Prophylactic ET_A antagonism prior to DSS exposure resulted in significant upregulation of *Plvap*, indicative of tighter endothelial barrier integrity (**Figure 4C**). While DSS treatment resulted in elevated *Cdh5*, which has been observed in affected tissue of IBD patients and rodent colitis[44], pre-treatment with Atrasentan prior to DSS exposure prevented this increase in *Cdh5* transcript expression from colonic barrier cells (**Figure 4D**). Shifting focus to transcripts of junctional proteins that are found in both epithelial and endothelial barriers, *Cldn1* was found to be upregulated in DSS animals whereas Atrasentan pre-treatment prevented this increase (**Figure 4E**). We did not detect any significant differences for the expression of *Ocln* or *Tjp1* (**Figure 4F, G**) between any of the treatment groups, though this may be due to the fact that animals were only exposed to 2 days of DSS. Taken together, these results indicate a pathogenic role of ET_A-mediated signaling in colitis development via barrier disruption. Prophylactic ET_A antagonism prevented both epithelial and endothelial barrier disruption and this appears to be mediated by both epithelial and endothelial junctional proteins.

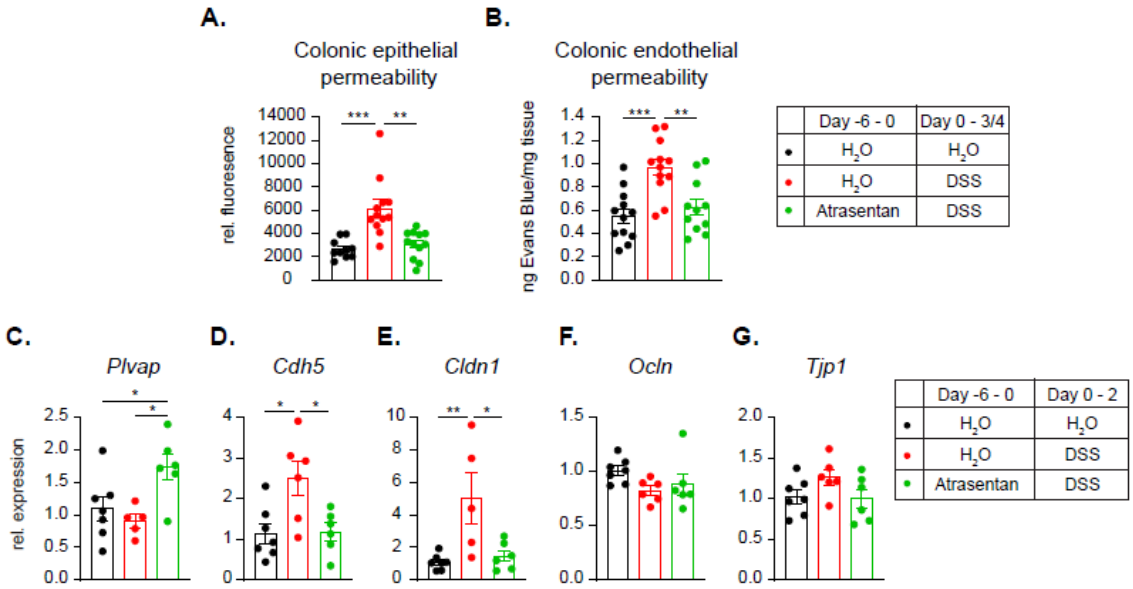

Figure 4. Prophylactic ET_A antagonism prevents DSS-induced disruption of colonic epithelial and endothelial barriers.

(**A**) Quantification of colonic epithelial barrier permeability via FITC-dextran assay displayed as relative fluorescence ($n \geq 10$). (**B**) Quantification of colonic endothelial barrier permeability via Miles assay displayed as ng Evans Blue/mg of tissue ($n \geq 11$). Fold change in *Plvap* (**C**), *Cdh5* (**D**), *Cldn1* (**E**), *Ocln* (**F**), *Tjp1* (**G**) transcript expression from colonic barrier cells of water control, DSS, and ET_A pre-treated DSS mice relative to water controls ($n \geq 5$). All transcripts were initially normalized to *Tbp*. Error bars represent mean ± SEM. Asterisks denote significance from Kruskal-Wallis followed by Dunn's multiple comparison test (A) or one-way ANOVA followed by Tukey's multiple comparisons test (B-G) at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Data are compiled from two (C-G) or three (A, B) individual experiments.

DISCUSSION

In this study, we examined how the microbiota impacts intestinal expression of different components of the endothelin system and how signaling via ET_A mediates intestinal pathogenesis. We demonstrate for the first time that the microbiota regulates expression of endothelin receptors within the colon. While our data suggests that the microbiota also regulates colonic expression of *Edn1/2*, this regulation is less clear as microbial reconstitution of adult gnotobiotic mice failed to alter intestinal transcript expression of ET peptides. While other studies have recognized a protective role for ET receptor antagonism with colitis, our data specifically suggests that this protection is the result of maintained barrier integrity when ET_A signaling is prevented. Our results also indicate that signaling via ET_A, but not ET_B, predominantly facilitates colotic pathology. Our study demonstrates a clear regulatory connection between the microbiota and intestinal expression of the endothelin system. Additionally, we reveal a role for ET_A-mediated signaling in contributing to colitic pathophysiology via barrier disruption.

As previously mentioned, Joe et al. demonstrated an important regulatory relationship between the microbiota and vascular physiology; hypotensive GF animals exhibit normotensive blood pressure with microbial reconstitution[27]. As blood pressure is dependent on vascular tone[45], and microbial reconstitution corrects hypotensive blood pressure, it would appear that the microbiota plays a crucial role in regulating vascular tone. Our findings expand on this topic regarding the regulatory relationship between the microbiota and the vasculature. We demonstrate a connection between the microbiota and

colonic *Ednra/b* expression, where intestinal *Ednra/b* is significantly higher in the absence of a microbiota. Additionally, we show that microbial conventionalization of gnotobiotic mice was able to return colonic *Ednra/b* back to expression levels observed in SPF mice. The microbiota also likely regulates colonic *Edn1/2* expression, though this regulatory relationship appears to be more complex relative to the ET receptors, as microbial reconstitution of adult GF mice failed to restore colonic *Edn1/2* expression back to levels observed in SPF mice. Moreover, differences in transcript expression between 3- and 8-wk old animals of the same background (SPF or GF) suggests that there is another component, in addition to the microbiota, that is temporally regulating intestinal expression of these endothelin system components. Alternatively, the discrepancy between colonic expression of ET components at these different time points could potentially be explained by the specific time point chosen for early-life assessment. Three-weeks of age represents a time point when mice are being weaned – thus, the transition from breast milk to solid food could be impacting colonic expression of the ET system, independent of the presence of a microbiota. Signaling via the ET receptors directly impacts vascular tone[45], thus it is likely that the microbiota is influencing intestinal vascular tone, at least in part, via regulation of intestinal endothelin receptor expression.

Our results exhibiting protection from colitis with the use of endothelin receptor antagonists is supported by previous studies demonstrating prevention of intestinal damage with ET receptor antagonism and various colitis models[12-15]. Our study demonstrates that prophylactic endothelin receptor antagonism is sufficient to prevent DSS-induced colitis, whereas other studies utilized treatment regimens of ET receptor antagonism beginning with colitis induction[12,14] or prophylactic ET receptor antagonism and continued

antagonism concurrently with colitis induction[13]. We additionally demonstrate a potential mechanism by which ET_A antagonism prevents colitis. We showed that prophylactic ET_A antagonism prior to DSS exposure resulted in the maintenance of both colonic epithelial and endothelial barrier integrity which were compromised in animals exposed to DSS without ET_A pre-treatment. Furthermore, we demonstrate that this protection of barrier integrity is likely mediated by junctional proteins.

Acute DSS exposure (2 days) was sufficient to observe changes in gene expression of junctional proteins from colonic barrier cells. Previous research has identified elevated expression of claudin-1 – a junctional protein found in both epithelial and endothelial cell-to-cell contacts – in affected tissues of IBD patients[46,47]. This elevated expression is tightly correlated with inflammation and indicates an overall deregulation of intestinal epithelial cell homeostasis[47]. Transcript expression of junctional protein claudin-1 was elevated with just 2 days of DSS, while pre-treatment with the ET_A antagonist prevented *Cldn1* upregulation. We also detected elevated gene expression for the vascular-specific adherin junctional protein, VE-cadherin (*Cdh5*), with DSS. Elevated intestinal VE-cadherin has been demonstrated with colitis[44] and is likely reflective of inflammation-induced pathologic angiogenesis – leading to immature, leaky blood vessel formation with chronic inflammation[6]. As with *Cldn1*, prophylactic ET_A antagonism prevented elevated expression of *Cdh5* in colonic barrier cells. Most strikingly, we observed elevated *Plvap* in colonic barrier cells of ET_A antagonist pre-treated animals, indicating enhanced endothelial barrier integrity. PV-1, the protein encoded by *Plvap*, is critically important for barrier integrity of diaphragm-covered fenestrated endothelium – of which endothelial type is found in the intestine[43]. In fact global knock out of *Plvap* in mice resulted in premature

death from edema and severe enteropathy, where the highest amount of vascular protein leakage was detected in the intestine[48]. Taken together, our data suggests that ET_A-mediated signaling induces colonic barrier disruption via alteration of cellular barrier junctional proteins early within the disease state. Prophylactic ET_A antagonism not only prevents barrier disruption and alteration of barrier junctional proteins, but may actually enhance vascular barrier integrity via upregulation of *Plvap*.

In summary, our study revealed that the microbiota regulates expression of the ET system in the colon, further implicating microbial involvement in mediating physiologic vascular tone within the intestine. Further, we demonstrate ET_A involvement in IBD pathogenesis via epithelial and vascular barrier disruption and altered gene expression of junctional proteins from colonic barrier cells. Prophylactic ET_A antagonism prevented DSS-induced colitis by maintaining and perhaps even enhancing barrier integrity. Prophylactic ET_A antagonism prevented DSS-induced alterations in both endothelial-specific (*Cdh5*) and nonspecific (*Cldn1*) junctional protein transcripts from colonic barrier cells. Further, prophylactic ET_A antagonism resulted in elevated gene expression of PV-1, suggestive of enhanced endothelial barrier integrity. This ET_A-mediated pathology in colitis represents a promising avenue for therapeutic development. While further research is necessary to uncover the therapeutic potential of ET_A antagonism during active disease, our data suggests at the very least that ET_A antagonism represents a promising therapeutic regimen for maintaining remission in IBD patients.

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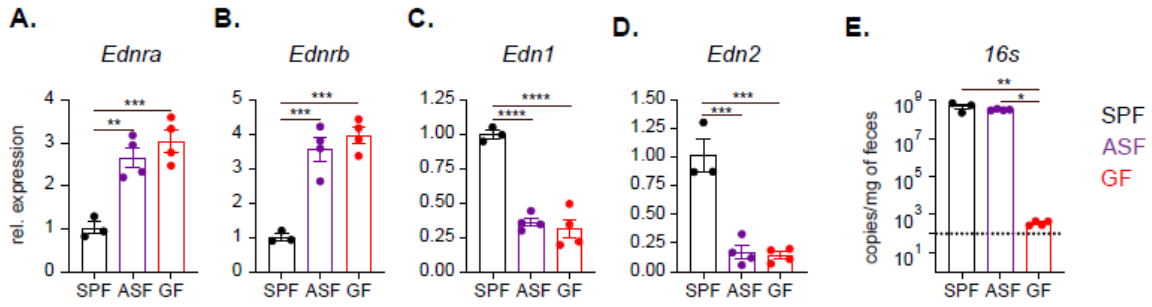

Figure S1. Specifically-defined minimal microbiota is insufficient to restore colonic endothelin system expression to SPF levels.

Fold change in *Ednra* (**A**), *Ednrb* (**B**), *Edn1* (**C**), *Edn2* (**D**) transcript expression from epithelial-denuded colonic tissue of specific pathogen-free (SPF), altered Schaedler flora (ASF), and germ-free (GF) C57/Bl6 mice relative to SPF ($n \geq 3$). All transcripts were initially normalized to TATA box binding protein (*Tbp*). (**E**) Fecal 16S rRNA copy number. Error bars represent mean ± SEM. Asterisks denote significance from one-way ANOVA followed by Tukey's multiple comparisons test at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Data are from one experiment.

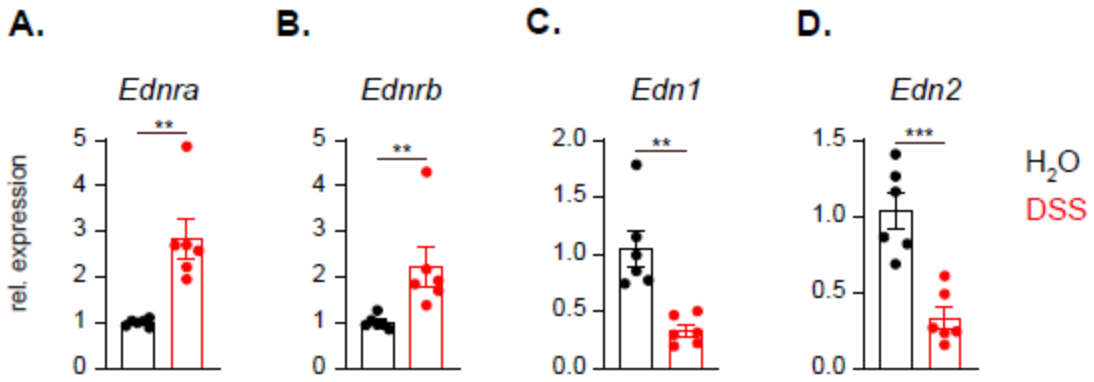

Figure S2. Colitis upregulates colonic gene expression of endothelin receptors and downregulates colonic gene expression of endothelin peptides.

Fold change in *Ednra* (**A**), *Ednrb* (**B**), *Edn1* (**C**), *Edn2* (**D**) transcript expression from epithelial-denuded colonic tissue of water control and DSS mice relative to water controls ($n \geq 6$). All transcripts were initially normalized to *Tbp*. Error bars represent mean ± SEM. Asterisks denote significance from Student's T-test at $p < 0.01$ (**), and $p < 0.001$ (***). Data are compiled from two individual experiments.

DISCUSSION

The multifactorial nature of IBD etiology classifies it as a complex disease. IBD develops at the interplay of genetic susceptibility, environmental exposures, microbiome alterations, and breach of the intestinal barrier – resulting in aberrant immune activation and imbalanced effector/regulatory cell populations. A combination of these "hits" will culminate in the eventual clinical manifestation of disease. The complexity of IBD makes it impossible to link a singular molecular mediator or gene to the disease phenotype. Even so, investigation into specific genetic culprits or molecular mediators provides invaluable insight into IBD pathophysiology. Fitting with the multifactorial nature of IBD, my thesis research focused on two separate factors that can impact IBD onset. First, we demonstrated how early life stress as an environmental trigger can lead to worse disease outcomes in IBD. Secondly, we revealed ET_A as a molecular mediator of IBD pathogenesis – demonstrating ET_A involvement in regulating colonic barrier integrity.

ELS Alters the Mucosa-associated Immune Cell Balance and Leads to Reduced Systemic Corticosterone

The Crohns and Colitis Foundation recently identified pyschosocial stress as 1 of 5 environmental triggers of great research interest[12]. Stress has long been anecdotally noted as an exacerbating factor in IBD, so much so, that IBD used to be considered a psychosomatic disorder[12,81]. Despite the fact that stress has been recognized as a triggering and exacerbating factor in IBD disease course, along with the fact that it is considered as a

determinant in disease relapse[82,83], very little is known about how stress mechanistically contributes to IBD pathophysiology. Even less is known about how stress contributes to the onset of IBD.

Given that early life stress is associated with numerous chronic inflammatory diseases and autoimmune disorders[35,38], we focused on how ELS may be impacting the onset and severity of IBD. We showed that ELS resulted in an altered mucosa-associated imune environment; demonstrating an initial deficit of anti-inflammatory IL-10-producing $CD4^+$ T cells, followed by a subsequent increase in pro-inflammatory IFNγ-producing $CD4^+$ T cells. The data suggests that ELS results in delayed differentiation of mucosal IL-10-producing $CD4^+$ T cells, an important population for the maintenance of intestinal homeostasis[134]. This delayed accumulation of IL-10-producing $CD4^+$ T cells consequently led to elevated Th1 effector cells – shifting the balance of mucosal immune cells. Intestinal homeostasis depends on a delicate balance between regulatory and effector immune cells and a dysregulated immune cell landscape is a hallmark of IBD[135]. Our results demonstrate ELS-induced imbalances in mucosa-associated immune cells – tipping the balance toward an inflammatory tone and ultimately enhacing susceptiblity to exacerbated inflammation upon colitogenic insult.

Consistent with previous studies[136], we also showed that ELS results in HPA-axis dysfunction. Both human and animal studies report contradictory effects of ELS on the circulating concentration of GCs, with elevated GCs observed in some settings[36,137] and decreased GCs observed in others[138,139]. We consistently observed decreased circulating CORT in both of the ELS animals models utilized within this study. While I do not believe that the reduced concentrations of circulating CORT was a main factor contributing to our

disease phenotype, it likely did play a role in mediating prolonged inflammation upon colitis induction. CORT is a potent anti-inflammatory molecule, acting on multiple immune cell subsets to mediate the resolution of inflammation[65]. Consistently depleted circulating CORT could result in failure to control inflammation and delay healing[140].

The Impact of ELS on Local Colonic Corticosterone Synthesis, Prolonged Intestinal Inflammation, and Altered Gene Expression of GC-related Targets

We demonstrated for the first time that ELS leads to a sustained impairment in colonic CORT production in the absence of any other perturbing stimuli. Upon colitis induction, we observed that ELS resulted in sustained intestinal inflammation in two separate models of ELS. We also verified that colonic CORT concentrations remain diminished during active colitis in ELS-exposed animals relative to controls. Despite the sustained deficit in colonic CORT observed with ELS alone, we did not detect differences in colonic transcripts for the GCR (encoded by *Nr3c1*) or Lrh-1 (encoded by *Nr5a2*), the receptor responsisble for intestinal CORT synthesis. This indicates that the basal reduction of CORT in the absence of colitis is likely not due to reduced GCR and thus reduced signaling capability, though impaired GCR functionality cannot be ruled out with this study. Additionally, these results indicate that this basally impaired local CORT is also not mediated by a loss of Lrh-1 expression.

Despite the fact that colonic GCR and Lrh-1 transcripts were not different between ELS and control mice under steady state conditions, assessment of these factors in an inflammatory setting revealed inflammation- and ELS-dependent alterations. In the context of intestinal inflammation, ELS resulted in reduced colonic expression of *Nr3c1* and *Nr5a2* relative to unstressed counterparts. Further, inflammation in non-stressed mice resulted in

the upregulation of colonic *Cyp11a1*, which is involved in *de novo* steroidogenesis. Both models revealed impaired *Cyp11a1* upregulation in ELS-exposed animals with colitis. The combination of decreased colonic *Nr5a2*, impaired upregulation of *Cyp11a1*, and diminished local CORT in colitic ELS animals strongly suggests that ELS leads to impaired intestinal steroidogenic induction upon inflammation. Further, the decreased colonic *Nr3c1* suggests a reduced capacity for GCR-mediated anti-inflammatory signaling. Reduced CORT and a reduced signaling capability likely additively contribute to the chronicity of colitis observed in ELS-exposed animals.

Our data further linked the prolonged colonic pathology in ELS-exposed animals to elevated TNF. Consistent with previous observations[135], we showed that colitis resulted in elevated intestinal Th1 and Th17 cells and increased colonic transcripts of pro-inflammatory cytokines IL-1β and IL-6. However, across two separate ELS models and multiple replicates, TNF stood as the sole pro-inflammatory factor that was consistently elevated in ELS mice relative to their non-stressed counterparts. Despite this, we were unable to identify a single cellular source responsible for the excessive TNF production. Rather, it is likely that the elevated TNF reflects an overall net-effect of impaired CORT and GCR expression – leading to poorly controlled TNF production from multiple pro-inflammatory cellular sources.

Elevated TNF Perpetuates Diminished Colonic Corticosterone Output

Following colitis induction in ELS-exposed animals, our data revealed a strong association between impaired local CORT, elevated local TNF, and prolonged intestinal pathophysiology. A complex, incompletely understood regulatory relationship exists

between TNF and GCs. Activated GCR facilitates anti-inflammatory effects mainly through direct interaction with pro-inflammatory-inducing transcription factors such as NF-κB and AP-1. Likewise, NF-κB and AP-1 – which are potently induced by TNF – can impede GCR signaling by interfering with the transactivation capability of the GCR[68]. Aside from the antagonistic effect of TNF on GCR, TNF can also amplify the GCR response – exhibiting a negative feedback loop to terminate inflammation[141,142]. However, this negative feedback capability appears to be absent in our study as ELS animals exhibited elevated intestinal TNF concurrently with decreased CORT and intestinal GCR by transcript. Further complicating matters, conflicting interactions between TNF and CORT specifically within the gut have been described. TNF administration during acute colitis can trigger intestinal CORT synthesis to reduce disease[143]. Another study found that TNF was responsible for reduced intestinal CORT via downregulation of *Nr5a2* in a chronic colitis model[144].

Our data along with the fact that our colitis model was more reflective of a chronic regimen, led us to further investigate the relationship between TNF and crypt-enriched colonic epithelial cells – the cellular fraction responsible for intestinal CORT synthesis[74]. TNF, IL-1β and IL-6 are known to activate the HPA-axis to induce GC production[141] and could perhaps regulate GC synthesis in other extra-adrenal GC-producing organs. Given this, we assessed transcript expression for the corresponding receptors of these cytokines within colonic crypt-enriched epithelial cells. Independent of ELS-exposure, inflammation resulted in a downregulation of transcripts for the IL-6 and IL-1β receptors, indicating that signaling via these receptors was not likely mediating impaired CORT synthesis. However, we observed significant upregulation of transcripts for both TNF receptors in colonic crypt-

enriched epithelial cells from ELS-exposed mice with colitis relative to their unstressed counterparts – indicative of an increased capacity for TNF signaling within this cellular fraction. Additionally, we demonstrated that TNF exposure of colonic crypt-enriched epithelial cells generated from ELS-exposed and control mice resulted in reduced CORT output from these cells. The effect of TNF on CORT output was more significantly impaired in colonic crypts cells of ELS origin relative to control mice. The elevated transcript expression of both TNF receptors along with the enhanced reduction of CORT output upon exposure to TNF suggests that colonic crypt epithelial cells of ELS-exposed animals exhibit enhanced TNF receptor signaling capability, ultimately contributing to diminished CORT synthesis.

Future Directions: Considerations of the Impact of ELS on Epigenetic Alterations, the Enteric Nervous System, and the Intesitnal Microbiota

Our study clearly demonstrates that ELS impairs local intestinal CORT output, both basally and during intestinal inflammation. While we did not definitively elucidate the mechanism whereby ELS mediates this impaired colonic CORT production, our data along with previously published literature[145,146], point to epigenetic changes as a probable mechanism. Much of the ELS-related literature has focused on how ELS alters brain physiology and function. This brain-centric research on ELS is understandable considering that 2 of the 3 sites of the stress-responsive HPA-axis are located in the brain. However, our study has clearly established the importance of exploring the impact ELS may have on other tissue sites.

Previously published data from both animal and human studies, have uncovered epigenetic alterations in multiple stress-response genes across various regions in the brain. These epigenetic alterations were found to impact GC output and alter the functional signaling capability of the GCR[145,146]. Though it has long been accepted that ELS results in HPA-axis dysfunction, which was validated in our model by decreased systemic CORT, we have shown for the first time that ELS also results in impaired CORT output in the colon. Given this, it is highly likely that the epigenetic changes observed in the brain with ELS, may also be occurring in other tissue sites, such as the intestine. Furthermore, our *in vitro* data on primary crypt-enriched epithelial cells from ELS colons exhibiting enhanced sensitivity to TNF stimulation resulting in further inhibition of CORT output, suggests that these crypt epithelial cells retain an imprinted signature (or epigenetic alteration) from ELS that is responsible for this increased sensitivity to TNF and enhanced blunting of CORT production. Taken together, review of the colonic epigenetic landscape from ELS-exposed animals represent a promising research direction for further understanding how ELS is impacting colonic CORT synthesis and enhanced susceptibility to prolonged intestinal inflammation.

Further areas of research interest for how ELS may enhance susceptibility to exacerbated intestinal pathophysiology include exploration of the enteric nervous system (ENS). With more than 100 million neurons and 400 million glial cells, the ENS represents the largest peripheral nervous system outside of the brain[147]. Intrinsic to the gut, the ENS is involved in various GI functions, including motility, secretion, absorption, visceral sensing, and immune and epithelial barrier function[148,149]. Despite the fact that the ENS may independently regulate GI function, the ENS still communicates with the central

nervous system (CNS) via input from both sympathetic and parasympathetic connections[149]. Given the close interaction between the CNS and ENS – termed the gut-brain axis – it seems highly plausible that ELS may impact typical ENS function. Moreover, the ENS exhibits a high degree of plasticity and is subject to substantial developmental changes in early postnatal life[147], indicating that the ENS is likely sensitive to alterations via exposure to ELS. In fact, studies utilizing neonatal maternal separation have found that these animals exhibit increased cholinergic enteric nerves[150] as well as elevated mucosal nerve fiber density and synaptogenesis in the GI tract[151]. Taken together, further investigation into how ELS may be affecting the ENS represents a promising research topic to enhance our understanding of IBD development and disease course in ELS-exposed individuals.

Lastly, further investigation into ELS-induced alterations of the microbiota and how that may impact colitis susceptibility and severity is warrented. Early life is a cruicial developmental window for the microbiota and it has been demonstrated that ELS alters typical microbial composition[152-155]. Perturbations to "normal" colonization could profoundly impact health throughout life.

Regulatory Relationship of the Microbiota on Colonic Gene Expression of the Endothelin System

In this study, we demonstrated for the first time that the microbiota impacts transcript expression of the ET system within the colon. The presence of a microbiota resulted in significant downregulation of colonic transcripts for both ET receptors, which was verified with microbial conventionalization of previously GF mice. Interestingly, the presence of a microbiota inversely impacted colonic transcript expression for ET-1 and

ET-2, exhibiting significantly higher expression in SPF mice relative to GF. Unlike *Ednra/b*, microbial reconstitution failed to alter colonic transcripts for *Edn1/2*, making the relationship between the microbiota and intestinal *Edn1/2* less clear. It is possible that the microbiota must be established early in life in order to observe microbial-induced changes in colonic *Edn1/2* expression. Regardless of the discrepency between colonic expression of transcripts for ET receptors vs ET peptides with microbial renconstitution, direct comparisons between SPF and GF mice reveal clear differences in colonic transcript expression for ET receptors and peptides – indicating a regulatory impact of the microbiota on intestinal expression of the ET system. Joe et al. recently reported that the microbiota is criticial for vascular physiology[156] and additionally demonstrated that hypotensive GF animals exhibit physiologocally relevant blood pressure upon microbial conventionalization[157]. Given that blood pressure is dependent on vascular tone and signaling via the ET receptors directly impacts vascular tone[94], suggests that microbial regulation of colonic *Ednra/b* may be influencing intestinal vascular tone.

Prophylactic ET_A Antagonism Prevented DSS-induced Barrier Disruption in the Colon

Despite the fact that previous studies have revealed protective effects of ET receptor antagonism in colitis models[131-133,158], the mechanism of action remains unclear. We verified this protection from colitis with prophylactic ET receptor antagonism, demonstrating that antagonism of ET_A specifically was sufficient to observe these protective effects. These results implicate signaling through ET_A in the mediation of IBD pathogenesis. We also showed that prophylactic ET_A antagonism prevented DSS-induced disruptions in both epithelial and endothelial barrier integrity. Further, pre-treatment with

the ET_A antagonist prevented DSS-induced alterations of colonic barrier cell transcripts for vascular endothelin-cadherin (VE-cadherin) and claudin-1. Atrasentan pre-treatment also resulted in increased transcript expression of PV-1 within the colonic barrier cells, indicative of increased endothelial barrier integirty. Taken together, the data suggests that ET_A antagonism prevents colitis by maintaining and perhaps even enhancing colonic barrier integrity likely through the preservation of barrier junctional proteins (**Figure 1**).

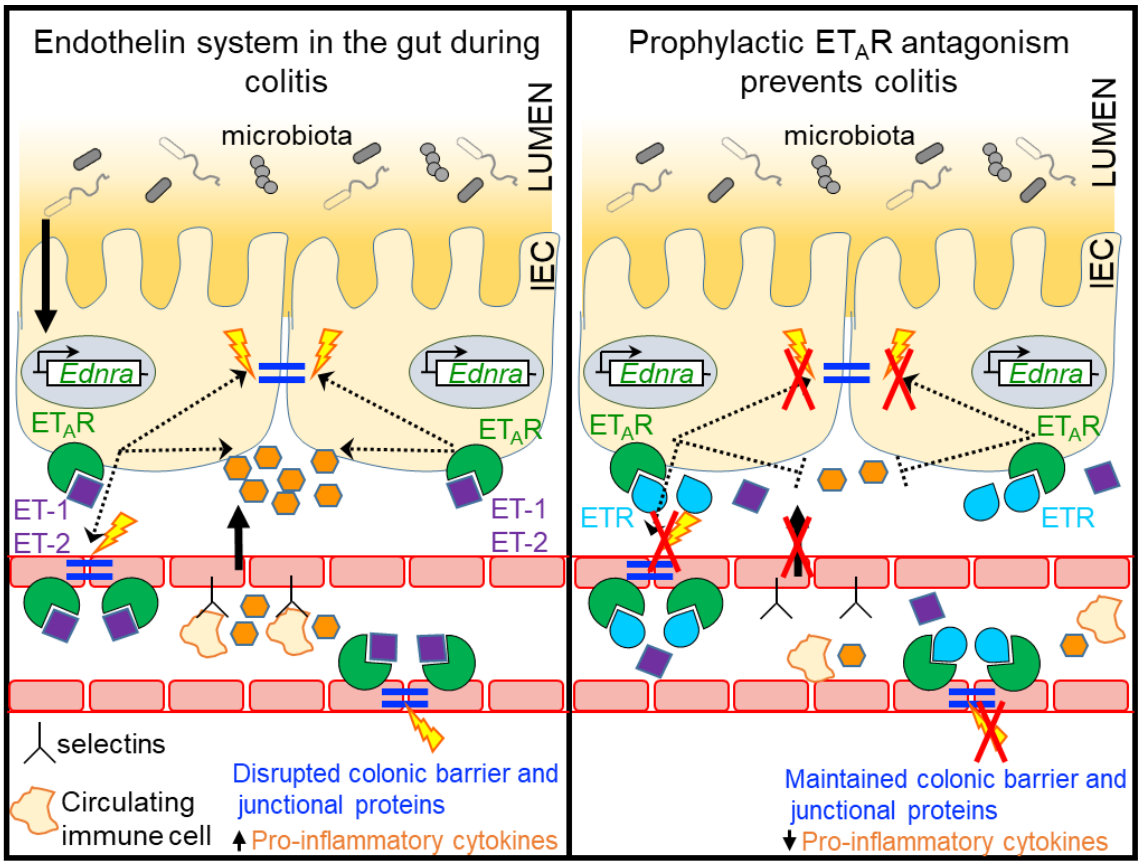

Figure 1. Proposed model for prevention of colitis development with ET_A antagonism. Our data suggests that signaling via ET_A contributes to the development of colitis upon a colitogenic insult. (**Left**) We propose that ET_A signaling contributes to loss of colonic barrier integrity via disruption of junctional proteins in both the epithelium and endothelium. Additionally, ET_A signaling within an activated endothelium will increase recruitment of circulating immune cells to the site of inflammation and thus further contribute to the amount of pro-inflammatory cytokines within the inflammatory environment. (**Right**) Prophylactic ET_A antagonism prevents colitis development by maintaining barrier integrity and homeostatic gene expression of junctional proteins. Further, ET_A antagonism, alongside the prevention of colitis development, results in little to no recruitment of/or extravasation of circulating immune cells to the colon.

Future Directions: In-Depth Microbiota Analysis and Investigation of the Relationship between Hypoxia, NO, and the ET system in Colitis Development

Our study demonstrates a regulatory role of the microbiota on colonic gene expression of the ET system. While we show this relationship by direct comparison of GF and SPF mice, along with microbial reconstitiution of gnotobiotic mice, we did not identify specific microbes or microbial metabolites responsible for regulating colonic ET expression. Fututre studies aimed at defining specific microbial regulators of colonic ET expression could reveal potential therapuetic targets for managing vascular complications that manifest in GI-related disorders. Additionally, investigation of ET system expression at peripheral sites may provide further insight as to whether microbial regulation of ET system expression is specific to the colon, or if it more broadly influences ET expression throughout the body.

Another promising investigative avenue includes the interplay between NO, hypoxia, and the ET system in colitis. Physiologic hypoxia within the gut is localized to the apical side of the epithelium. With colitis, hypoxia drastically increases and can be detected throughout the mucosa[113]. Hypoxia potently induces ET-1 expression[159] and excessive ET-1 can decrease endothelial NO by suppressing expression of endothelial nitric oxide synthase (eNOS)[160]. Hatoum et al. demonstrated that microvascular dysfunction in affected tissue biopsies of IBD patients was facilitated by a loss of NO-mediated vasodilation[161]. This was later demonstrated to be caused by decreased eNOS activity, leading to uncontrolled oxidant formation[162,163]. Thus, further investigation into the specfic connection between hypoxia, ET_A, and loss of NO responsiveness in IBD may reveal mechanistic targets with therapuetic potential.

Overall Summary

Within this thesis I first demonstrate a connection between ELS and IBD susceptiblity and severity. ELS resulted in diminished intestinal CORT and exhibited an altered mucosa-associated immune environment, both of which could increase susceptibility to worse outcomes upon colitogenic insult. I show that ELS-exposed animals do in fact exhibit prolonged intestinal inflammation that appears to largely be driven by significant increases in local TNF concurenntly with impaired intestinal steroidogenesis and diminished capacity for GCR-mediated signaling.

Secondly, I show that the microbiota regulates gene expression of the ET system in the colon. Additionally, we reveal that prophylactic ET_A antagonism prevents colitis by maintaining the integrity of both the epithelial and endothelial barriers. Gene expression analysis of colonic barrier cells suggests that prophylactic ET_A antagonism maintained barrier integrity by preventing colitis-induced alterations of junctional proteins. Further, the increased transcript expression of PV-1 suggests that ET_A antagonist pre-treatment may even enhance endothelial barrier integrity. Collectively, the data presented here within suggests that ET_A signaling contributes to IBD pathogeneis via colonic barrier disruption.

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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

MEMORANDUM

DATE: 11-Mar-2022

TO: Pollock, Jennifer

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 11-Mar-2022.

Protocol PI:	Pollock, Jennifer
Title:	Molecular Mechanisms of Early Life Stress and Risk of Inflammatory Bowl Disease
Sponsor:	Crohn's & Colitis Foundation of America
Animal Project Number (APN):	IACUC-21214

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 21-Apr-2024.