CHRONIC PSYCHOLOGICAL STRESS AS A MODULATOR OF INTESTINAL MUCOSAL IMMUNITY

CHRONIC PSYCHOLOGICAL STRESS AS A MALADAPTIVE IMMUNOMODULATOR OF INTESTINAL MUCOSAL IMMUNITY IN THE CONTEXT OF CROHN'S DISEASE

By

LENA DARWISH, B.Sc.

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AUTHOR: Lena Darwish, B.Sc. (McMaster University)

SUPERVISOR: Dr. Brian K. Coombes; Dr. Dawn M.E. Bowdish

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LAY ABSTRACT

Crohn's disease (CD) is a type of inflammatory bowel disease driven by genetics, the environment, and the intestinal microbiome. In Canada, the rates of CD are among the highest in the world, representing an urgent health concern. Among the environmental risk factors, chronic stress has been reported to trigger and worsen CD flares through mechanisms that are not fully understood. My work investigates how chronic stress affects the intestinal microbiome and immunity to create a disease-state environment which worsens CD. Using a mouse model of chronic stress, I have determined that stress promotes the activation of inflammatory pathways in the gut and alters the composition of the gut microbiome within the small intestine. This research will help to understand how stress impacts intestinal inflammation and therefore improve CD management strategies.

ABSTRACT

Crohn's disease (CD) is an inflammatory bowel disease driven by genetic, environmental, and microbial factors. CD presents a pressing health concern in Canada which has one of the highest prevalence and incidence rates of CD in the world. Chronic psychological stress has been reported as an environmental risk factor for CD which can provoke flares after a period of quiescence and/or exacerbate inflammation. However, the mechanisms which underpin the relationship between stress and disease activity are not known. Previous work has shown that stress can exacerbate CD by disrupting the gut microbiome, leading to uncontrolled inflammation and dysbiosis. The **hypothesis** of my work is that chronic psychological stress is a maladaptive immunomodulator of the gut, compromising host interactions with the microbiota and thereby resulting in disease-state dysbiosis. I used a chronic variable stress (CVS) mouse model of chronic stress to probe the effects that stress has on host control of the microbiome as it relates to CD. In this model, I found that while CVS did not modify the structural integrity of the ileum nor the quantities of suspected leukocyte populations, CVS-exposed mice showed a transcriptional profile consistent with Th17 inflammatory activation. Moreover, sequencing of the ileal microbiome showed that CVS may be permissive for expansion of bacterial families originally present in low levels whilst contracting dominant families such as Muribaculaceae and Lactobacillaceae. Dysbiosis was also indicated by a significant increase in stool content of antimicrobial Lipocalin-2 (LCN2). This work will provide valuable insights into the risk factors which drive disease activity, thus informing the standard of care for CD.

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ABBREVIATIONS AND SYMBOLS

AIEC ..... adherent-invasive *Escherichia coli*
CD ..... Crohn's disease
CFU ..... colony forming units
CVS ..... chronic variable stress
DSS ..... dextran sodium sulfate
EDTA ..... ethylenediaminetetraacetic acid
ELISA ..... enzyme-linked immunosorbent assay
FBS ..... fetal bovine serum
GALT ..... gut-associated lymphoid tissue
GC ..... glucocorticoid
GI ..... gastrointestinal
GR ..... glucocorticoid receptor
HPA ..... hypothalamic-pituitary-adrenal
IBD ..... inflammatory bowel disease
IEC ..... intestinal epithelial cell
IL- ..... interleukin; indicates type of cytokine
ILC3 ..... innate lymphoid cell type 3
LCN2 ..... lipocalin-2
LPMC ..... lamina propria mononuclear cell
MLN ..... mesenteric lymph node
PBS ..... phosphate-buffered saline
RNA ..... ribonucleic acid
rRNA ..... ribosomal ribonucleic acid
SLO ..... secondary lymphoid organs
Stress ..... psychological stress
Th ..... T helper cell; indicates T cell type
TLR ..... toll-like receptor
TNFα ..... tumour necrosis factor α

DECLARATION OF ACADEMIC ACHIEVEMENT

Lena Darwish and Brian K. Coombes conceptualized the project. Lena Darwish, Brian K. Coombes, and Dawn M.E. Bowdish designed experiments. Lena Darwish performed experiments. Lena Darwish, Megan T. Zangara, and Katarina R. Iacobucci sacrificed animals. Lena Darwish analyzed the data.

INTRODUCTION

CROHN'S DISEASE

Overview of Crohn's disease

Crohn's disease (CD) is a type of inflammatory bowel disease (IBD) that can present anywhere along the gastrointestinal (GI) tract, manifesting as patches of inflamed tissue interpolated with regions of non-inflamed tissue[1]. CD follows a relapsing and remitting course that progressively deteriorates the intestinal barrier with each bout of active inflammation, giving rise to symptoms such as abdominal pain, chronic diarrhea, weight loss, and fatigue. As the disease advances in severity, it is estimated that half of patients develop complications that require surgical intervention such as bowel obstructions, fistulas, abscesses, and cancer. CD can also incite extraintestinal inflammation such as joint pain and skin rashes[1,2]. The course and clinical presentation of CD show considerable heterogeneity between patients. However, consistent trends in epidemiological data indicate that the global CD burden is concentrated in Western, high-income countries in North America, Western Europe, and Oceania[3]. As of 2023, Canada has among the highest annual incidence (30 per 100,000) and prevalence (410 per 100,000) of CD in the world[4]. In keeping with the association between the Western world and elevated CD burden, low-middle income countries are experiencing a trend of increasing CD incidence that is concomitant with the progressive "Westernization" and industrialization of these regions[3]. The epidemiology of CD suggests that the Western lifestyle entails environmental pressures that, when compounded with other predisposing factors, antagonize aberrant intestinal inflammation toward the development of CD.

The microbial signature of Crohn's disease

The microbial profile of CD patients points to a role for gut dysbiosis in disease etiology and progression. The microbiota is affected by internal and external factors such as treatment interventions, diet, disease status, age, ancestry, and myriad other variables[5,6]. Despite the heterogeneity in patient lifestyle and microbial composition, there are consistent hallmark features of the gut microbiome in CD. Dysbiosis associated with CD can be described as a reduction in overall diversity which enables certain bacterial taxa to expand while constricting others. Among the changes in bacterial families, CD patients generally have a significant reduction in the protective commensal Bifidiobacteriaceae family[6,7], and butyrate-producing strains within the Lachnospiraceae, Ruminococcaceae, and Clostridiaceae families[7–9]. Microbe-derived butyrate serves a regulatory and anti-inflammatory role in maintaining intestinal homeostasis, to the extent that the abundance of butyrate is significantly correlated with the success of clinical remission using frontline anti-tumour necrosis factor (TNF) therapy[10]. CD patients also show an enrichment in opportunistic species within the Bacteroidaceae family, a group of commensals which can cause inflammation in a compromised host[7]. Most relevant to this work, CD patients often show a significant enrichment of genera within the Enterobacteriaceae family, specifically *Escherichia*[6,7]. At a high taxonomic level, common changes in the CD-associated microbiome can be summarized by a significant reduction in the Actinobacteria and Firmicutes phyla coupled with an enrichment in the Bacteroidetes and Proteobacteria phyla.

In many CD patients, the outgrowth of Enterobacteriaceae is coupled with an encroachment on the intestinal mucosa, a unique feature that distinguishes these bacteria as a new CD-associated *Escherichia coli* pathotype called adherent-invasive *E. coli* (AIEC)[11–14]. The pathogenic potential of AIEC is dependent on the host intestinal environment. Immune and microbial homeostasis allow AIEC to exist innocuously in the gut as evidenced by its presence in

a small subset of non-IBD individuals[13]. AIEC can bloom and invade deeper intestinal sites by leveraging perturbations in immune and microbial niches, exploiting the intestine as a reservoir for replication. Mouse models have identified environmental exposures that potentiate AIEC expansion in the gut leading to dysbiosis, barrier damage, and inflammation. Interestingly, many of the factors which permit AIEC blooms overlap with known CD risk factors, including antibiotic exposure, high fat diet, gastrointestinal infection, and acute psychological stress[15–19].

Another important perspective to consider is the composition of the microbiome preceding clinical manifestation of CD, as it sheds light on the elements that prime the gut for disease – a theme that will be explored throughout the current study. A recent study performed within The Crohn's and Colitis Canada Genetic Environmental Microbial (GEM) project used machine learning in a prospective cohort study of the microbiome of healthy first-degree relatives of CD patients, showed that the pre-disease composition of the microbiome is associated with subsequent onset of CD in these patients[20]. Overall, the pre-disease "microbiome risk score", referring to disease liability, showed that increased abundance of mucin-degrading bacteria in the gut was associated with increased microbiome risk scores for CD, whereas abundance of butyrate-producing species from the Lachnospiraceae and Ruminococcaceae families decreased the microbiome risk score, emphasizing the protective role of butyrate[20]. Both host and bacterial metabolites, such as bile acids and SCFAs, comprise the intestinal metabolome which has been found to have divergent profiles in healthy individuals compared to the pre-disease phase and during disease[6,7,20,21], which are reflective of microbial perturbance. Finally, it must be noted that while bacteria are the focus in the microbial etiology of CD, the gut microbiome is composed of protozoa, fungi, viruses, and other microbial elements which are under-researched in the field and should be considered as focal points of future work.

Crohn's disease "activity" and its predictors

Although the causative events that precipitate and subsequently dictate the course of CD are not exactly defined, the epidemiological and genetic data indicate that the etiology of CD is decidedly multifactorial. The oscillation of CD between relapse and quiescence is referred to as disease "activity", wherein increased disease activity indicates an interval of heightened clinical inflammation. Thus, the consensus is that CD activity is governed by a complex network of genetic, microbial, and environmental factors that collectively dysregulate intestinal mucosal immunity[1].

The heritable component of CD is demonstrated by the increased familial risk of CD among the relatives of patients[22,23]. Indeed, genome-wide association studies (GWAS) have found over 200 CD-associated risk loci[24,25]. Though CD has been one of the largest beneficiaries of the development of GWAS, the findings on the origin of CD are indeterminate as genetics alone are not sufficient to instigate IBD; one study summarized that the sole contribution of genetic risk loci accounts for just 13.1% of CD liability[25]. Hence, the compounding role of environmental elements and the microbiota have become a major point of interest in delineating the full scope of CD pathogenesis, especially given the striking clustering of IBD in the West.

Many environmental elements are implicated as triggers that exacerbate and/or precipitate CD. Diet is among the most researched risk factors because the ingestion of food introduces a deluge of antigenic stimulation that effects change in both the host and the microbiota. In human and animal studies, the Western diet (high fat; low fibre; ultra-processed) has been scrutinized for its detrimental effects on the intestinal barrier[26]. Increased barrier permeability, immune cell infiltration, dysbiosis, and microbial encroachment are the primary intestinal pathologies that are elicited in mice consuming diets rich in emulsifiers (e.g. maltodextrin and polysorbate-80), fat, or sucrose[18,27,28]. The mechanisms by which certain dietary elements modulate the gut are not well-

defined, but it is clear that the gut microbiome is a critical mediator as pathology is attenuated in germ-free mice supplemented with a typical pro-inflammatory diet[27]. Moreover, the role that microbial perturbation plays in disrupting gut homeostasis is emphasized by the association of antibiotics and gastrointestinal infections with IBD etiology. Early life exposure to antibiotics is posited to reshape the microbiome at a critical phase in development such that the gut acquires a durable susceptibility to CD[29]. The mechanisms that underpin the increased risk of CD post-antibiotics are not clear but appear to be driven by dysbiosis of the gut microbiome[30]. In fact, antibiotics have been shown to directly potentiate the expansion of CD-associated bacteria within the gut[16]. It is worth noting that some of the risk factor potential that is ascribed to antibiotics in human observational studies might be compounded by the gastroenteric infection that commonly precedes a course of antibiotics[31], thus warranting more detailed studies to further disentangle these variables.

Research on the environmental determinants of disease continues to expand toward a more comprehensive understanding of socioeconomic risk factors. The findings consistently reinforce the fact that mental and physiological health are inextricable. Psychological stress, hereafter referred to as “stress”, has long been observed to provoke and exacerbate IBD flares; IBD was even inaccurately classified as a psychosomatic disorder in the 1950s[32]. The subjective nature of stress makes it a difficult risk factor to reliably study in humans. A common method is to assess stress levels by quantifying “perceived stress” as described by patients over acute or chronic timelines using Cohen’s Perceived Stress Scale (CPSS) or analogous questionnaires[33]. Comprehensive demographic information and statistical methods are also used to distill stress as an independent variable that influences CD activity. Indeed, disease severity indices show that perceived stress is a statistically significant predictor of CD activity in patients within both acute and chronic timescales[32,34–37].

THE NEUROIMMUNE AXIS AND STRESS

The anatomy of the neuroimmune axis

The brain can exert immune modulation in peripheral immune sites through transduction of signals via two main pathways: the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (ANS)[38]. This communication is bi-directional, in that physiologic changes at peripheral immune sites can relay signals up the brain-immune axis and modulate brain activity. However, to underscore the discussion on the role of psychological stress in disease, the unidirectional brain-to-periphery cascades are the most pertinent to review.

All organ systems are connected to the central nervous system through peripheral innervation. Direct neurological communication to peripheral immunity can occur along ANS neurocircuits that innervate primary and secondary lymphoid organs, including the bone marrow, spleen, and gut-associated lymphoid tissue (GALT)[39]. In rodent studies, the neural geography associated with different pathways of immunomodulation has been determined with polysynaptic retrograde viral tracing, wherein pseudo viruses expressing reporter genes can illustrate multiple neurocircuits originating from the site of viral infection[40,41]. Rodent studies that have inoculated any of the bone marrow, colon, spleen, or peritoneum with a pseudo virus collectively showed that the brain-immune axis runs along the spinal cord and permeates the forebrain, midbrain, and hindbrain, including the medulla, ventral tegmental area (VTA), locus coeruleus (LC), hypothalamus, amygdala, hippocampus, insular cortex, and the septal and motor cortices[40–42]; in essence, the limbic system and its ancillary structures which are collectively responsible for emotional processing and neuroendocrine regulation[43]. Human functional magnetic resonance imaging (fMRI) studies have alluded to similar patterns of neurocircuitry by showing that the activity of the limbic system is tunable by peripheral inflammation[44–47]. Though neurocircuits in

the brain-gut-immune axis are mediated by myriad neurotransmitters and cytokines, the pertinent effector molecules within the context of the stress response in both rodents and humans are the catecholamines norepinephrine (NE) and epinephrine (EPI), and acetylcholine (ACh)[39,48].

Catecholamines comprise the sympathetic branch of the ANS, better known as the "fight-or-flight" response, and they bind to cell surface adrenergic receptors[48]. Leukocytes which express these adrenergic receptors are susceptible to immunomodulation by NE, particularly in the gut which is the second-most innervated organ in the human body; in fact, the enteric nervous system (ENS) is recognized as a distinct arm of the ANS, constituting the gut-brain axis[48]. The ENS is connected to the brain by the vagus nerve, and its neurons interact with the intestinal immune system under both normal and diseased conditions. For example, muscularis macrophages (MM) reside around the myenteric plexus portion of the ENS, which is a unique geographic positioning in comparison to other gut macrophages[49,50]. MMs highly express the β_2 adrenergic receptor (β_2AR) which facilitates integration into ENS signaling pathways[49,50]. In normal conditions, MM maintain bi-directional communication with the ENS to facilitate homeostatic functions such peristalsis through the microbe-dependent release of bone morphogenic protein-2 (BMP-2)[51]. Under infection, tyrosine hydroxylase-expressing neurons within the ENS are activated, thus producing NE which binds to MM cell surface β_2AR and polarizes MMs toward a unique polyamine-mediated protective program to limit neuron loss[49,50].

ACh is the main neurotransmitter in the parasympathetic branch of the ANS, better known as the "rest-and-digest" response, and it binds to nicotinic and muscarinic receptors expressed on the target cell surface[48]. Among its immunological functions, ACh is best known for facilitating the "cholinergic anti-inflammatory pathway"[52], wherein it regulates tumour necrosis factor (TNF)-mediated inflammation by binding to nicotinic receptors expressed on macrophages[53].

The HPA axis is a critical neuroendocrine regulatory system which maintains systemic physiological processes and constitutes the second arm of the brain-immune axis. At homeostasis, the HPA axis orchestrates a delicate pattern of pulsatile cortisol release according to a circadian rhythm, which is coordinated through a cascade of GC action[38]. The perception of a real or imagined threat triggers an emotional stress response driven by the limbic system. In the brain, the paraventricular nucleus of the hypothalamus releases corticotropin releasing hormone (CRH) to communicate the stressor to the anterior pituitary gland, thereby stimulating the release of adrenocorticotropic hormone (ACTH) which travels through the bloodstream to the adrenal glands to elicit the release of cortisol into circulation[38]. The pattern of cortisol release is maintained by clock genes which dictate circadian rhythm and is regulated in response to stressors that disrupt its rhythmicity by a negative feedback loop between the hypothalamus and blood cortisol levels[38].

The regulatory effects of GCs are pleiotropic, but most of all, GCs are potent immuno-modulators across all levels of immune function[54]. Once released into circulation by the adrenal glands, GCs are carried to the organ systems where they freely diffuse across the cell lipid membrane to bind to cytoplasmic glucocorticoid receptors (GRs), specifically GR-α[54]. The activated GC-GR complex then enters the nucleus to effect physiological change by binding to GC response elements (GREs) in the DNA. GRE accessibility differs depending on cell type, hence the heterogeneity of GC potency across different cells. GR-β is an alternatively spliced GR form that does not directly interact with GCs, but it binds to DNA potentially as a negative regulator of GR-α by preventing its access to GREs[54]. Once in the nucleus, the GC-GR complex can influence gene expression by recruiting chromatin remodelling machinery, tethering of essential immune transcription factors such as NF-κB and STAT3/5/6, stimulation of inflammatory gene mRNA degradation, and more[54]. Under normal conditions, GCs maintain

immune homeostasis and attenuate inflammation by tightly regulating the expression of cytokines, chemokines, adhesion molecules, and receptors[54].

Psychological stress is an adaptive response and a pervasive comorbidity

Across a spectrum of diseases, including CD, individuals that undergo periods of stress can experience adverse health effects[38,55]. Though stress has been long observed to dysregulate immune function, the machinations that underlie the relationship between stress and disease are not well-defined. As the brain perceives stress, it effects physiological change along the HPA axis and the ANS through the release of GCs and catecholamines, respectively, which act on their cognate receptors[54,56].

The immunomodulatory capacity of GCs has been leveraged to create frontline treatments for CD and other inflammatory diseases[54]. However, GCs represent a double-edged sword as they can also impart defects across many compartments of immunity, as evidenced by health complications in stressed individuals and negative outcomes of long-term steroid therapy[54]. Rodent models have demonstrated that unfavourable apoptosis of lymphocytes, inappropriate immune profile skewing, and impediment of humoral immunity are some of the defects associated with excessive levels of endogenous or exogenous GCs[19,57–61]. Immunomodulation by stress is context-specific as each cell type has a pre-determined degree of sensitivity to GCs, which the assay for transposase-accessible chromatin coupled with high-throughput sequencing (ATAC-seq) has found to be governed by chromatin accessibility to the GR[62,63].

Stress can be categorized into two types that differ in duration: acute and chronic. Acute stress and chronic stress both represent a disruption of homeostasis, but they each possesses a distinct mode of immunomodulation[38,64].

Acute stress: a brief overview

Acute stress is an adaptive response to immediate threats by way of precipitating transient physiological changes that are geared toward survival[38]. The most common model of acute stress in rodents entails physical restraint in a ventilated conical tube. Acute restraint stress studies in rodents have shown that the early stages of acute stress are characterized by fulminant lymphocyte, monocyte, and neutrophil mobilization from their home reservoirs, allowing for trafficking of these cells to lymphoid and peripheral organs in later stages[56,60]. This urgent immune cell redistribution is advantageous, but comes at the expense of collateral immune dysregulation. In a rodent model of acute stress, GC-mediated migration of lymphocytes from the periphery and secondary lymphoid organs (SLOs) to the bone marrow can protect against the acquisition of autoimmunity in experimental autoimmune encephalomyelitis (EAE)[60]. The caveat is that the diversion of adaptive immunity from SLOs undermines the immune surveillance at these sites. Mice that were subjected to acute stress during the early phases of SARS-CoV2 or influenza A virus (IAV) infection had significantly higher viral titres in the lung, worsened lung pathology, and increased mortality[60]. The underlying stress-induced defect was characterized as inadequate humoral immunity marked by smaller mediastinal lymph nodes lacking in lymphocytes, therefore blunting the anti-viral immune response[60]. Human studies recapitulate the leukocyte mobilization and redistribution under acute stress that are both GC- and catecholamine-based[65,66].

The purpose of immune modulation under stress is not known. Stress-induced immune modulation may simply be a collateral outcome of the redirection of resources to other "fight-or-flight" processes, but it also may be an evolved immune reconfiguration that directly contributes to survival. Increased activation of natural killer (NK) cell-mediated cytotoxicity is consistently observed as a consequence of acute stressors ranging in intensity from tandem-skydiving, to

public speaking, to timed brain teasers[67–69]. NK cells possess an elusive susceptibility to modulation under stress, and the consequences in otherwise healthy individuals are not known.

Adverse modulation by acute stress is also associated with asthma and allergic disease. Patients with atopic dermatitis or psoriasis show a significantly exaggerated mobilization of NK cells in response to acute stress in comparison to healthy subjects, suggesting that individuals with pre-existing immune perturbation may be more susceptible to immunomodulation by acute stress[70], and a mouse model of asthma subjected to acute stress showed a GC-dependent increase in the levels of interleukin (IL)-6, IL-9, and IL-13, which are classical asthma-associated cytokines that suggest priming toward asthma[71]. Lastly, a consistent trend in GC-based suppression of innate immunity under acute stress has also been observed, including the impairment of the phagocytic function of granulocytes and monocytes, decreased response to stimulation of IL-8 and TNFα release[72], and suppression of the MYD88 and toll-like receptor (TLR) cascade[68].

Acute stress in humans can offer context-specific immunological advantages, most prominently the augmentation of humoral immune response. Exposure to acute mental stress immediately before immunization with an influenza vaccine stimulates an increased induction of virus-specific IgG in women, but not in men, at 4 and 20 weeks post-vaccination[73]. Conversely, exposure to acute mental stress immediately before immunization with a Meningococcal A+C vaccine enhanced the Meningococcal serogroup A IgG response in men, but not in women, at 4 weeks post-vaccination[74]. The mechanisms behind acute stress-induced enhancement of immunization and the potential sexual dimorphisms within this phenomenon are not clear.

Most relevant to the current study, we have found that acute restraint stress in a mouse model of AIEC colonization permits AIEC to opportunistically bloom throughout the GI tract, leading to dysbiosis, inflammation, and a damaged mucosal barrier[19]. The deficit was based in

GC-mediated apoptosis of a heterogeneous $CD90^+$ population within the ileum that is comprised of T helper 17 (Th17) and type 3 innate lymphoid cells (ILC3), which are chiefly responsible for IL22-mediated barrier protection and maintenance[19]. The consequences of acute stress in the ileum bring forth the ileum as a new site of interest within stress research, and raise the question of the ability of chronic stress to facilitate a disease-state microbiome. Previous studies on stress in the context of IBD demonstrate that chronic stress can maladaptively modulate intestinal immunity within the colon in ways that are divergent from acute stress, but the implications for the gut microbiome and disease are not yet clear.

Chronic stress: a maladaptive immunomodulator

Chronic stress is a maladaptive and prolonged disruption of homeostasis that is not evolutionarily favoured. Chronic stress is closely scrutinized as a disease risk factor as it is a pervasive component of human life, ranging from daily nuisances to long-term mental illness. The body is in a perpetual process of predictive adaptation termed "allostasis", which ensures physiological stability through constant fine-tuning in anticipation of stress[75]. Sustained allostasis results in the cumulation of physiological changes termed the "allostatic load"; as in, increased exposure to stress demands more resourceful and profound physiological reconfiguration, thus increasing the overall allostatic load[75]. The body can then reach a point of "allostatic overload", which is summarily described as an impasse where the cost of physiologically accommodating chronic stress outpaces an individual's tolerance for coping with stress[75]. At this tipping point, the HPA axis begins to unravel as cortisol production becomes recalcitrant to regulation by upstream checkpoints[76]. The state of allostatic overload is where chronic stress takes its most pathological form.

Chronic stress in humans and rodent models presents an immune state characterized by immunosuppression, dysregulation, and low-grade inflammation[38,54,77]. In comparison to controls matched for age and socioeconomic status, high perceived stress scores are associated with blood C-reactive protein (CRP) levels indicative of sustained low-grade systemic inflammation[78,79], significantly slower wound healing, and decreased sensitivity to stimulation of IL-1β release from leukocytes *in vitro*, suggesting a broad dysregulation of innate immunity[80]. Leukocytes isolated from chronically stressed individuals show conserved changes in their composition and transcriptional profile which are indicative of a pro-inflammatory immune milieu, which can be traced back to myeloid lineage cells[63,81,82].

The myeloid lineage is highly susceptible to modulation under chronic stress[83]. Chronically stressed individuals exhibit leukocytosis characterized by monocytosis and neutrophilia[63,81,82]. In fact, it has been suggested that the degree of leukocytosis has a positive relationship with the severity of major depressive disorder (MDD), a mental illness that is akin to chronic stress[84]. The transcriptome of blood leukocytes isolated from chronically stressed individuals demonstrate a conserved transcriptional response to chronic stress, which entails a significant upregulation of pro-inflammatory cytokines (*Il6, Il1β, Il8, Tnf*); innate immune activation of genes containing nuclear factor (NF)-κB response elements; inflammasome activity; cell proliferation; and defense against oxidative stress. Conversely, chronic stress significantly downregulated cell cycle regulators (*Tgf-β, Cdk* gene family); apoptosis genes; type I interferon (IFN) antiviral response elements; antimicrobial response elements; B lymphocyte function; and genes with glucocorticoid receptor response elements (*N3cr1*)[63,79,82,85]. Moreover, the transcriptional shift of blood leukocytes under chronic stress has been majorly ascribed to monocytes and dendritic cells[63,83,86,87]. These changes hold true in mouse models of chronic stress, and are corroborated by transcriptomic changes within the bone marrow that are consistent with

pro-inflammatory priming[63,82]. Overall, the conserved chronic stress response primes the hematopoietic compartment toward hyper-inflammation, unchecked cell proliferation, and oxidative stress, complemented by the suppression of antiviral and antimicrobial defense systems, defective apoptosis cues, and GC resistance.

In line with human observations, rodent models of chronic stress exhibit a profound leukocytosis that is characterized by monocytosis and neutrophilia in the blood[63,81,82]. Leukocytosis under chronic stress in mice is provoked by a combination of GC-dependent and β_3-adrenergic enhancement of hematopoiesis in the bone marrow, in part due to the impairment of CXCL12 production, a critical chemokine for regulation of hematopoietic stem cell (HSC) proliferation and liberation into the blood, leading to overproduction of myeloid progenitors[81,88]. As the HSCs migrate to the spleen, they are ushered into uncontrolled myelopoiesis by GC and β-adrenergic upregulation of myeloid growth factor GM-CSF, thus manifesting as monocytosis with concomitant splenomegaly[89]. Moreover, ATAC-seq has revealed that these changes are based in epigenetic reprogramming of monocytes and their bone marrow progenitors, showing that stress affects monocytes in a way that is akin to innate immune training[63]. The exposure to chronic stress incited a protracted production of splenic $CD11b^+$ myeloid cells for at least 24 days after the cessation of stress[89], which may be valuable data to help discern the lifetime ramifications of chronic stress. However, a gap remains in the knowledge about the durability and plasticity of transcriptional priming in monocytes after the cessation of stress.

Chronic stress is also correlated with changes in humoral immunity, wherein chronically stressed individuals show an impaired induction of humoral immunity 4-6 weeks after influenza vaccination[90–93]. This defect is marked by decreased Influenza IgG titres post-vaccination and decreased lymphocyte release of IL-2 and IL-1β *in vitro*, the latter showing an additional stress-induced impairment of cell-mediated immunity[91–93]. The mechanism behind the suppression of

humoral immunity is not clear. However, chronic stress in mice can hinder splenic B cell maturation and impair germinal centre formation[94,95], a histologically distinct structure where B cell clonal expansion, class switching, somatic hyper-mutation, and antibody affinity maturation take place. Corticosterone released under chronic stress abrogates germinal center expression of miRNA-155, a microRNA which is essential for regulation of processes within germinal centers, resulting in apoptosis of splenic B cells by ubiquitination and impairing the development of humoral immunity under stress[95]. This mechanistic insight into the defects imparted by corticosterone on humoral immunity may help explain the negative correlation between chronic stress in humans and the efficacy of vaccination.

GC resistance is a detrimental consequence of prolonged exposure to GCs resulting in the decreased immune sensitivity to attenuation by GCs, sustaining uncontrolled inflammation which leads to the disease-primed state[96,97]. Under sustained elevated GCs, the tightly regulated negative feedback loop governing the HPA axis unravels because of unresponsive GC-GR interaction, such that the correlation between the number of circulating leukocytes and plasma cortisol breaks down during chronic stress[97]. The molecular mechanisms of GC resistance represent a moving target, including: downregulation of GR-α expression and/or translation; GR post-translational modifications which decrease sensitivity; increased GR-β expression; defective chromatin remodelling; increased P glycoprotein efflux pump activity; and more[76,96]. Though GC resistance is typically researched in the context of exogenous GC therapies, persistent elevation of endogenous GCs under chronic stress is emerging as a risk factor for resistance[97].

IBD that is refractory to frontline corticosteroid therapy as well as patient dependence on corticosteroids are both manifestations of reduced sensitivity to GCs and occur in 20% and 36% of patients within the first year of treatment, respectively[98]. This brings to question the impact of precursory GC resistance resulting from chronic stress on the efficacy of corticosteroid therapies;

it would be interesting to explore the overlap between risk of corticosteroid-refractory disease and degree of perceived stress in human cohort studies. As previously mentioned, the knowledge on the molecular mechanisms of immuno-suppression by GC-based therapies is incomplete and has been vastly outpaced by the widespread clinical application of these drugs. The mechanisms by which chronic stress subjugates the immune system may inform the CD treatment in the context of stress-induced flares and GC-refractory disease.

Chronic stress murine models perturb the intestinal barrier

Impairment of the intestinal epithelium is the primary manifestation of psychological stress in the gut (Fig. 1). Human and rodent models of stress have shown an increase in gut transepithelial conductance, macromolecule leakage, bacterial adhesion to the epithelium, and bacterial translocation across the follicle associated epithelium[99–102]. Comparisons within these studies have highlighted that the epithelial defects associated with chronic stress were more pronounced and durable than acute stress. The overall increase in barrier permeability has been associated with inflammatory mast cell degranulation triggered by corticotropin releasing hormone (CRH) in humans and mice, though the full mechanism which drives barrier damage under stress remains unclear[103–105].

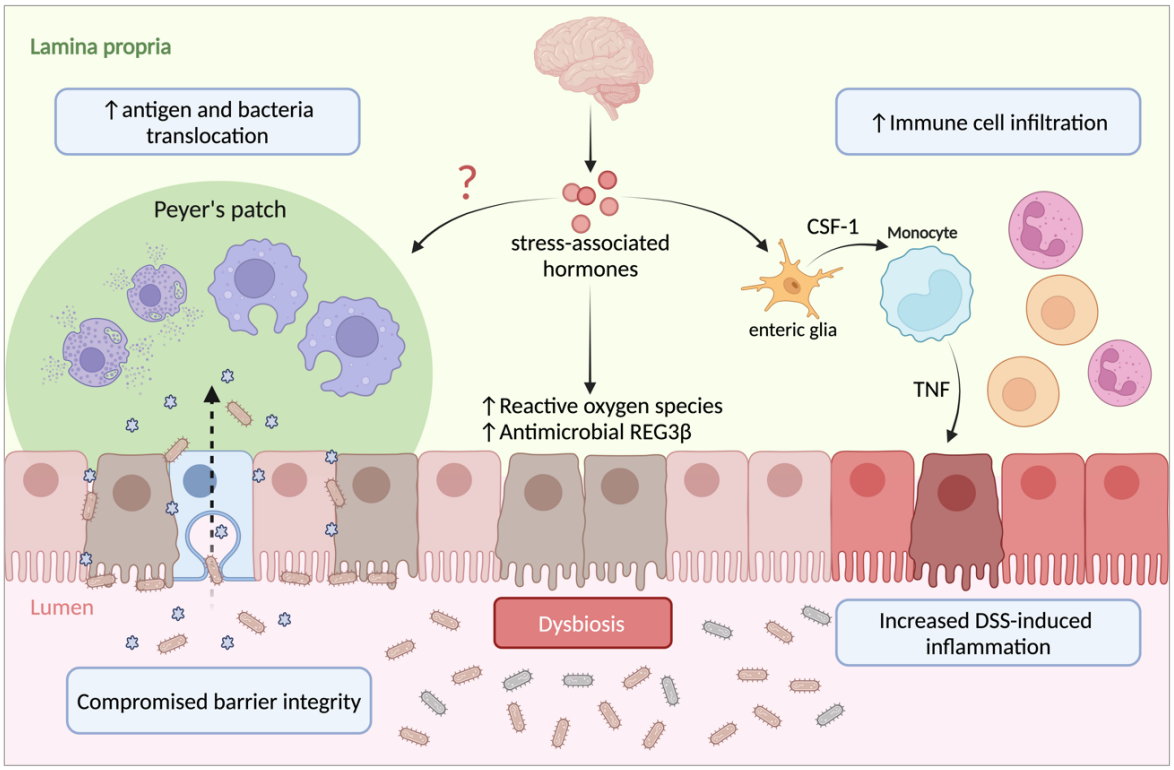


Figure 1 – Schematic of the known effects in rodent models of chronic stress on the intestinal barrier.

The barrier damage under stress is partly based in the modulation of intestinal epithelial cell (IEC) function. A model of chronic social stress showed a shift in colonic IEC gene expression toward inflammation and disruption of microbial homeostasis[106]. Relevant differentially expressed genes include *Il18* and *Saa1* (inflammatory response); *Nos2* and *Duox2* (oxidative stress response); and *Reg3b* (antimicrobial defense)[106]. These changes in gene expression under stress were dependent on the presence of the gut microbiota, such that chronic social stress in germ-free mice and antibiotic-treated mice did not show differential expression of the aforementioned genes[106]. In this model, stress modified the composition of the microbiome toward dominance of the Muribaculaceae family and catalase-positive bacteria, accompanied by diminishing catalase-negative microbiota. The catalase-positive bacteria have a competitive ability to overcome the stress-induced production of reactive oxygen species (ROS) via NOS2 and DUOX2, which may have permitted their expansion at the expense of catalase-negative niches[106]. The implications of these stress-induced changes in the gut microbiome are not known.

The physiological consequences of stress as it relates to IBD have become widely studied in mouse models. Chronic social stress for 19 days was found to induce epithelial damage, leukocyte infiltration, and release of TNFα and IFNγ[107]. Adrenalectomy to abrogate the HPA axis activation by chronic stress showed that these defects were, in part, mediated by GC-GR interaction. This study also compared the effects of acute and chronic stress, showing that serum GCs are elevated after acute stress and led to immuno-suppression, while chronic stress had a divergent immunomodulatory phenotype resulting in adrenal insufficiency and GC resistance which may be the basis of the observed aberrant colonic inflammation[107,108]. Chronic stress has also been studied in conjunction with established models of IBD by implementing chronic restraint stress followed by a dextran sodium sulfate (DSS) model of colitis[109]. Chronic restraint stress primed the colon for worsened DSS-induced colitis as shown by histological evaluation

indicating exacerbated barrier erosion[109]. Moreover, chronic restraint stress in combination with DSS resulted in neutrophil recruitment to the colon and increased overall circulating leukocytes[109]. Colonic neutrophil infiltration was similarly found in a study which paired a CVS model with DSS and showed that NE-mediated β-adrenergic signaling promoted neutrophil trafficking[110]. Mesenteric lymph nodes (MLNs) showed a significant decrease in the proportion of B cells and $CD4^+$ T cells, coupled with a significant increase of neutrophils and $CD8^+$ T cells[109]. Moreover, chronic stress changed the composition of the microbiome, resulting in a dominance of Firmicutes[109]. At the genus level, operational taxonomic units indicated an enrichment of bacteria that genetically clustered with inflammatory species such as *Helicobacter*, *Streptococcus*, and *Enterococcus*[109]. In line with a previous study[106], antibiotic treatment abrogated the modulatory effects of stress on DSS-induced colitis, pointing to an inextricable role for the microbiota in the pathophysiology of stress[109].

Lastly, the most recent seminal study by Schneider and colleagues applied a chronic restraint stress model to outline an axis along which stress signals are relayed between the brain and intestinal monocytes via the enteric nervous system (ENS), consequently worsening inflammation in a DSS model of colitis[111]. The model consisted of 3 hours of chronic restraint stress for 14 days, with DSS in the drinking water starting at day 7. Chronic restraint stress significantly exacerbated DSS-induced colitis as shown by increased colonic injury in histology, weight loss, and mortality[111]. Single cell RNA sequencing of colonic immune cells pointed to a pathogenic role for TNF-producing monocytes in stressed mice[111]. Indeed, monocyte depletion and chemical blocking of TNF protected stressed mice from the worsening of DSS-induced colitis[111]. The illustration of a novel communication cascade along the brain-gut axis was a salient contribution to the knowledge on stress in intestinal disease. In this cascade, stress-induced GCs from the adrenal gland act on enteric glia to promote their transition to enteric glia associated with

psychological stress (eGAPS), which release Colony Stimulating Factor 1 (CSF1). CSF1, in turn, acts on monocytes to promote their recruitment to the colon and TNF production, thus worsening intestinal inflammation under DSS[111].

Considerable progress has been made toward identifying the role of chronic stress in intestinal pathophysiology. However, the consequences of stress for the gut microbiome and host-microbe interactions are a novel avenue to explore in the determinants of CD activity.

Models of chronic stress in vivo

The study of chronic stress in rodents is a decades-old evolution of protocols which have adapted to changing laboratory animal ethics and increasingly quantitative definitions of stress. As it stands, the field has several mainstay chronic stress protocols which can be broadly divided into two categories: (1) the repetition of a single stressor over the course of 7-30 days; and (2) the use of a variety of mild unpredictable stressors for 14-30 days.

The accepted chronic stress protocols have co-opted acute stressors and apply these stressors for prolonged periods of time, either in various combinations or as a single repetitive "homotypic" stressor[112]. Common chronic stress protocols include restraint stress in a ventilated conical tube for several hours per day[111]; social defeat stress, wherein male rodents are placed in a cage with another unfamiliar male rodent, eliciting fear, aggression, and anxiety[113]; water exposure/avoidance stress wherein rodents are forced to swim or placed on a platform in a small body of water[114]; or a combination of several types of stressors termed chronic variable stress (CVS) or chronic mild unpredictable stress[115]. While the comparative efficacy of "homotypic" versus "heterotypic" stress is a subject of debate[112], CVS is advantageous to prevent rodent habituation to stressors, ensuring a continuous physiological stress response which is demarcated by the elevation of circulating corticosterone[115].

HYPOTHESIS AND AIMS

Chronic psychological stress has long been associated with worsened intestinal inflammation in IBD, showing chronic stress to compromise gut immunity and damage the epithelial barrier. The physiology of the intestinal barrier under stress has been scrutinized for decades. However, the persistent observation of dysbiosis under chronic stress has been overlooked and is elusive in its origin and consequences in individuals with CD. Moreover, the ileum is the most common site for CD-related inflammation, such that presentation of CD is ileocolonic in 19-45% of patients and strictly in the terminal ileum in 30-45% of patients as assessed by the Montreal classification system of CD[2,116,117]. Yet, the ileum has been understudied in *in vivo* models of disease in favour of the colon. Thus, **I hypothesize** that chronic psychological stress maladaptively modulates intestinal immunity within the ileum, permitting a disease-state dysbiosis which worsens inflammation. I have investigated three compartments of the host-microbe relationship under stress: *(i)* the microbial composition of the ileal microbiome in control and stressed groups with a temporal resolution; (*ii*) barrier integrity and the cellular landscape of mucosal immunity in the ileum as it relates to host-microbe interactions; and *(iii)* changes in the transcriptional profile within inflammatory pathways of interest.

To this end, I implemented a 21-day model of chronic variable stress (CVS) (Fig. 2)[81,115]. In this model, naïve 6-12 week old C57BL/6 mice, both males and females, were used in control (n = 4) and stressed (n = 4) groups. Mice were exposed to two stressors per day: once during the light cycle, and an overnight stressor throughout the dark cycle. The light cycle and dark cycle stressors were rotated daily to avoid habituation. Light cycle stressors included: 1h cage shake at 80 rpm; 15 min forced swim in 30°C water ; 3h social isolation; 1h restraint stress. Dark cycle stressors included: 45° cage tilt; damp bedding; light cycle disruption; food deprivation; water deprivation. Secondarily, I briefly delineated preliminary observations from the use of an

alternative chronic stress model involving repeated restraint stress (RRS) to evaluate the best route moving forward in the study of chronic stress and CD (Fig. 3).

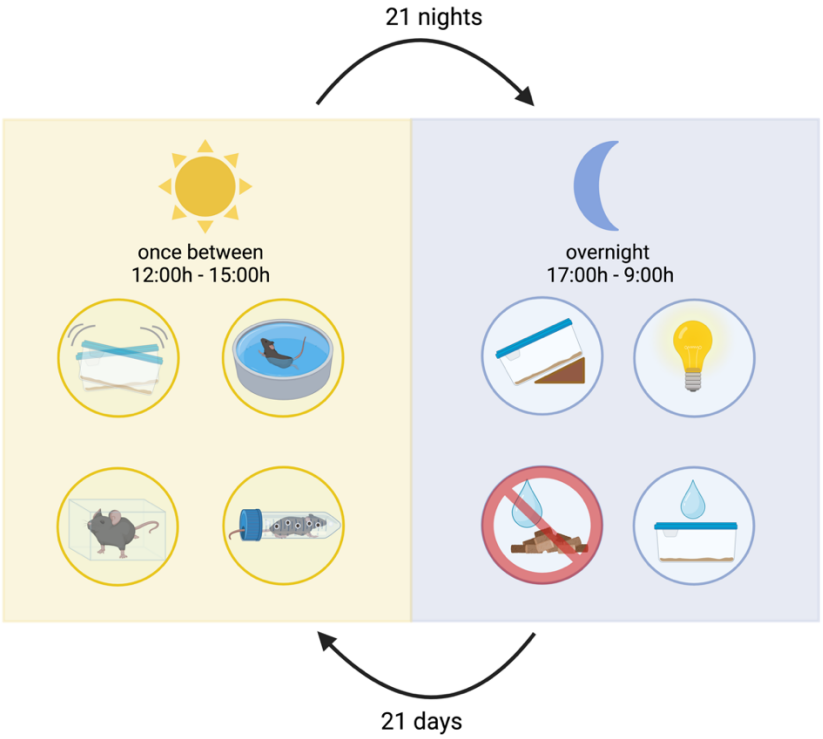


Figure 2 – Schematic of the chronic variable stress (CVS) protocol. A set of light cycle and dark cycle stressors are rotated daily such that the stress group (*n*=4) will be exposed to two stressors per 24h: one light cycle stressor and one overnight dark cycle stressor. The control group (*n*=4) will remain undisturbed. Light cycle stressors (left): 1h cage shake at 80 rpm; 15 min forced swim in 30°C water; 3h social isolation; 1h restraint stress. Dark cycle stressors (right): 45° cage tilt; damp bedding; light cycle disruption; food deprivation; water deprivation. CVS is applied for 21-24 days.

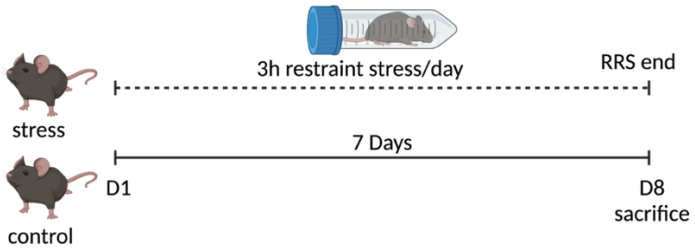


Figure 3 – Schematic of the repeated restraint stress (RRS) protocol.

RESULTS

Chronic variable stress does not cause overt illness or intestinal pathology

The chronic variable stress (CVS) mouse model was used to model chronic stress *in vivo* (Fig. 2). The CVS model applies a cycle of diverse stressors over the course of three weeks to avoid habituation and maintain a novel stress response[81,115]. Mice are exposed to a brief stress episode during the light cycle (7:00h-17:00h) and stress that is sustained overnight during the dark cycle[115]. The set of light cycle stressors include: 1h cage shake at 80 rpm; 15 min forced swim in 30°C water; 3h social isolation in a confined space; 1h restraint stress. Dark cycle stressors include: 45° cage tilt; damp bedding; overnight illumination; food deprivation; water deprivation.

Naïve male and female 6-12 week old C57BL/6 mice were subjected to the CVS protocol to examine the consequences of chronic stress on overall mouse health. Mice were monitored by weighing at baseline and every 3-5 days thereafter. CVS did not cause body weight stagnation or weight loss, regardless of sex (Fig. 4a,b).

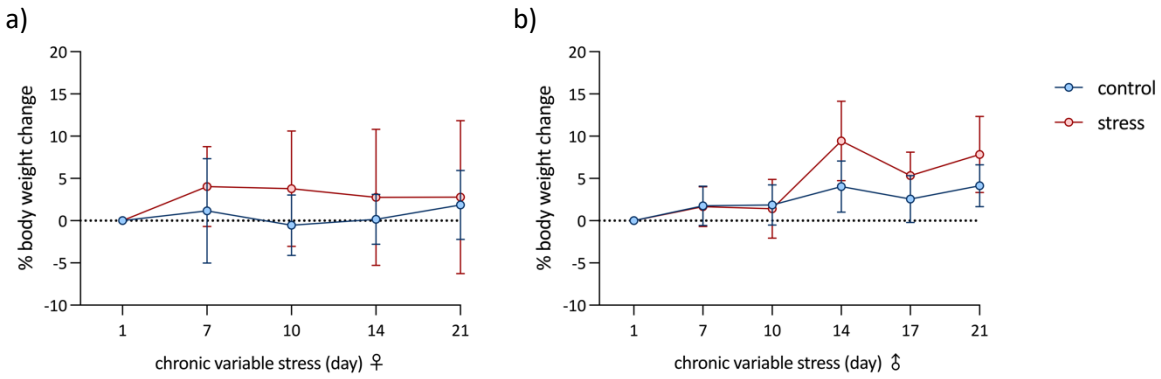
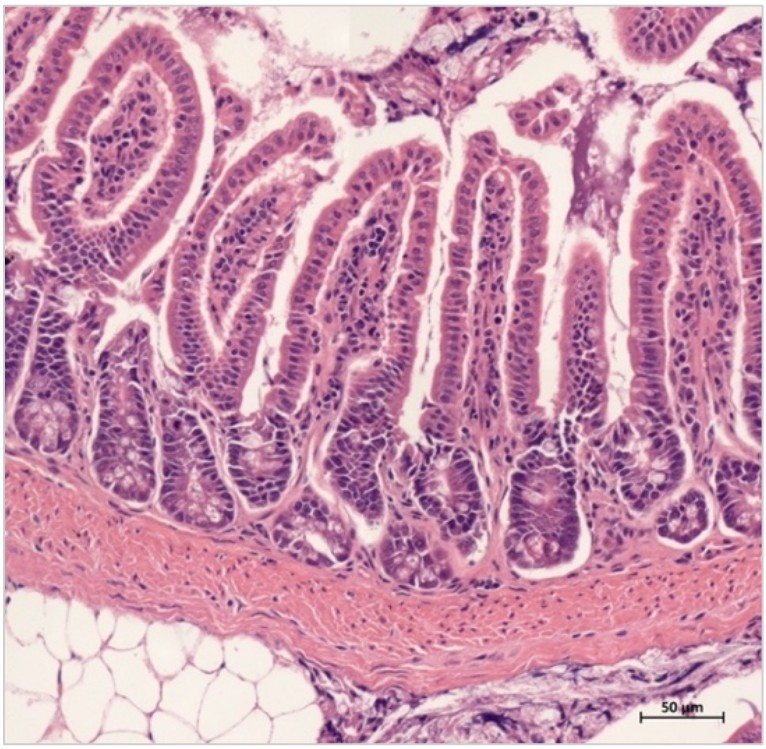


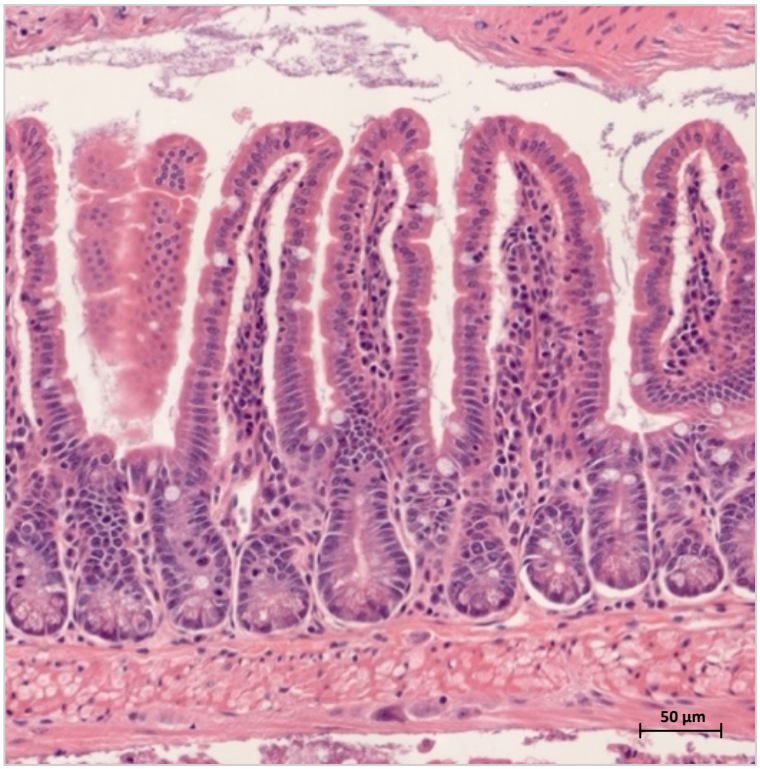
Figure 4 – Percent body weight change over three weeks of CVS. (a) females, control (*n*=4) stress (*n*=4). (b) males, control (*n*=4) stress (*n*=4). Error bars represent standard deviation.

The structural integrity of the intestinal barrier in stressed and control female mice was examined with histological analysis of the ileum, cecum, and colon. Hematoxylin and eosin (H&E) staining revealed that CVS does not compromise the structure or composition of the intestinal epithelial layer in the ileum and colon, nor does it elicit inflammation as indicated by the absence of tissue-infiltrating polymorphonuclear and mononuclear leukocytes (Fig. 5a,b). Overall, intestinal histology from both control and stressed groups were unremarkable.

a) **ILEUM**



CONTROL



STRESS

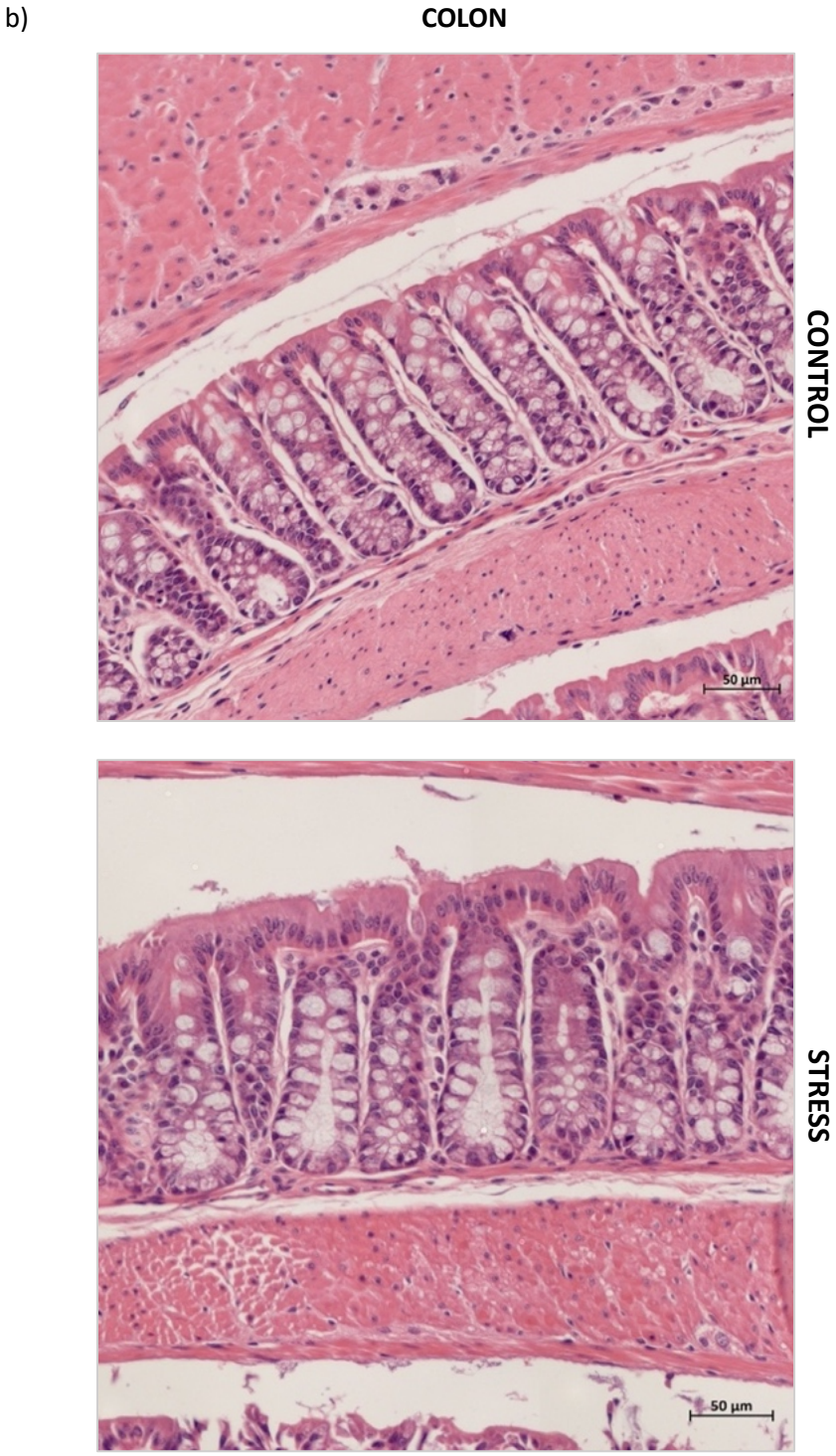


Figure 5 – Representative histological sections of H&E stained intestinal tissue from control and CVS mice. (a) Ileum: control (left) and stress (right). (b) Colon: control (left) and stress (right).

CVS activation of the HPA axis is sex-dependent

Stress in mouse models can be quantified by measuring plasma corticosterone, the dominant glucocorticoid in rodents which is elevated under stress[115]. Blood was drawn from mice on the last day of stress (D21) between 12:00-14:00h, which was kept consistent across experiments to control for circadian rhythmicity of corticosterone. Plasma separated from whole blood by centrifugation was analyzed for corticosterone concentration using liquid chromatography and mass spectrometry (LC/MS). Altogether, the data indicate that there was no significant change in the level of circulating corticosterone under CVS as compared to the control condition (Fig. 6a). However, stratification of the data by sex revealed that male mice subjected to CVS experienced a significant elevation of corticosterone in response to stress, while female mice were resistant to stress-induced corticosterone production and maintained levels similar to those of the controls (Fig. 6b). These data indicate a sex-dependent effect of activation of the HPA axis by CVS in mice.

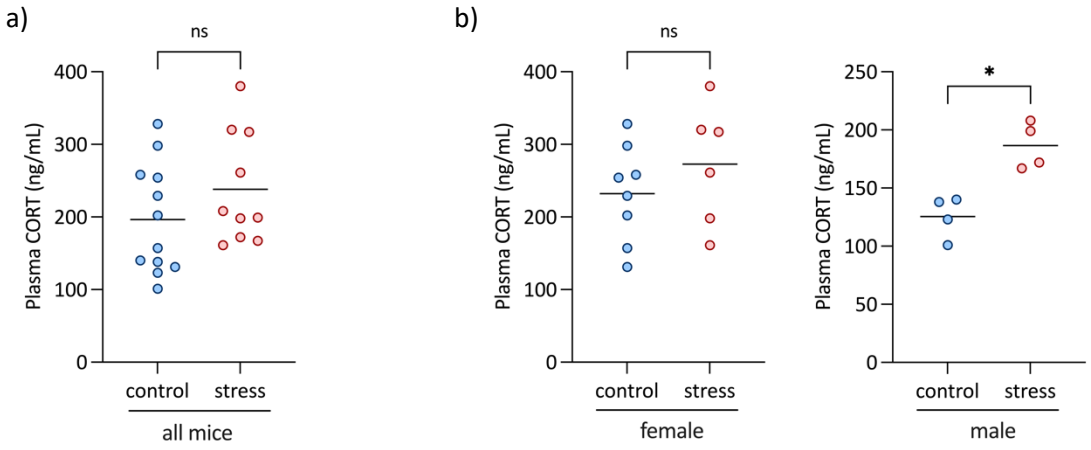


Figure 6 – Plasma corticosterone (CORT) level on day 21 of CVS. LC/MS was used to quantify plasma corticosterone level in control (male $n = 4$; female $n = 8$) and stressed (male $n = 4$; female $n = 8$) mice on Day 21 of CVS. (a) Plasma corticosterone quantity in both male and female mice. (b) Plasma corticosterone quantity stratified by sex. Significance was determined with a two-tailed Mann-Whitney test (ns=no significance; *P≤0.05).

CVS perturbs the composition and decreases the diversity of the bacterial microbiome in the ileum

Models of chronic stress in mice have been shown to alter the bacterial composition of the colonic microbiome or the microbial content of fecal pellets[106,109,118]. The ileum can be a primary site of CD-related inflammation, and as such, I chose to profile the ileal microbiome using a cohort study, wherein stressed and control mice were sacrificed at day 0 (baseline), day 14, and day 21 of CVS to capture temporal changes in the microbiome throughout CVS. After sacrifice, I retrieved the luminal contents of the ileum and the v3-v4 region of 16s ribosomal RNA (rRNA) was sequenced by Illumina sequencing.

The ileal microbiota of the control and stressed mice showed several differences in the day 14 and 21 timepoints. The control groups on days 0, 14, and 21 remained consistent in the composition of bacteria within the ileum, showing a dominance of the Muribaculaceae, Lactobacillaceae, and Clostridiaeceae families (Fig. 7). On day 14 of CVS, the stressed group showed a contraction of Muribaculaceae and Lactobacillaceae concomitant with the introduction of extensive blooms of Staphylococcaceae and Aerococcaceae into the microbiome (Fig. 7). The colonization by Staphylococcaceae under stress persisted until day 21 in half of the mouse cohort, while the Aerococcaceae family was diminished at this timepoint (Fig. 7). Notably, the mouse in the stressed group that had the most effective clearance of Staphylococcaceae on day 21 also showed a unique expansion in Lachnospiraceae. In addition, the Lactobacillaceae population in stressed mice recovered to levels of colonization similar to the control group by day 21, whereas the Muribaculaceae family remained significantly diminished (Fig. 7).

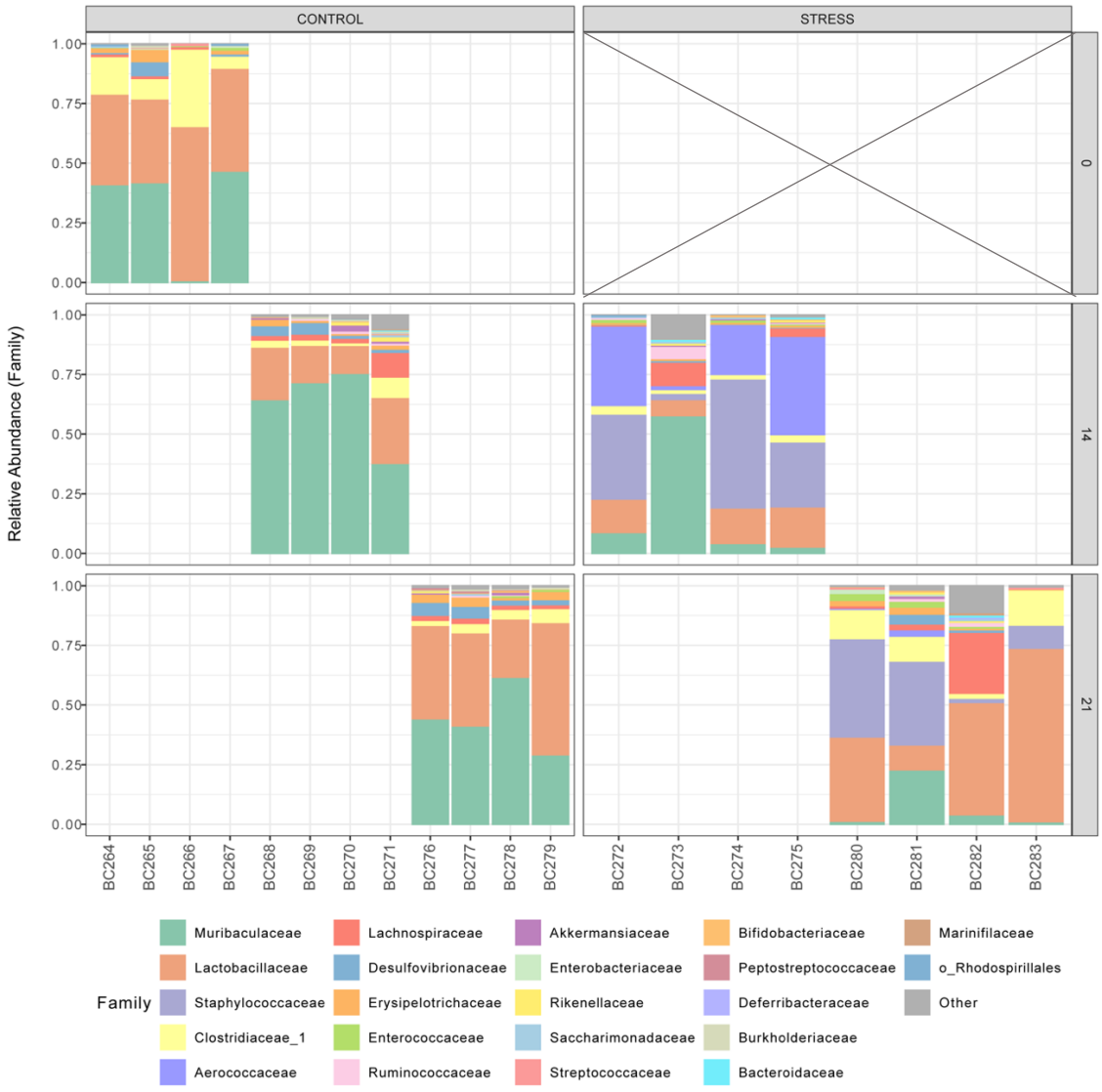
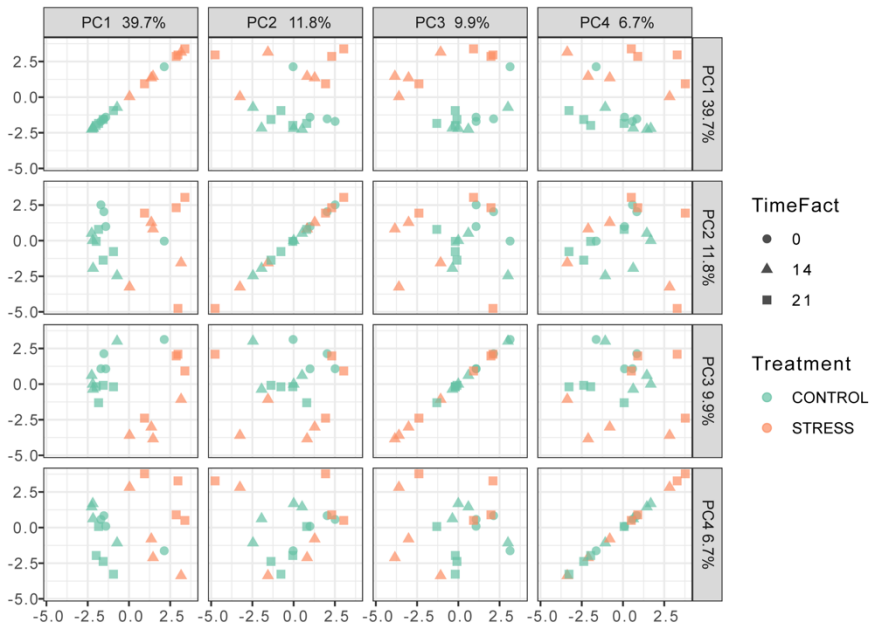


Figure 7 – Relative abundance of bacterial families in the ileum of control and stressed mice on day 0, day 14, and day 21 of CVS. Each pair of panels at 0, 14, and 21 represents a separate cohort of mice (control $n = 4$; stress $n = 4$). Each bar represents one mouse.

Principal component analysis (PCA) was used to evaluate inter-sample β-diversity to compare the quantities of bacterial families between samples in the control and stressed conditions and the three different timepoints (D0, D14, D21). The ordination plots show a clear segregation of control and stressed samples along the first principal component, suggesting that the quantitiative changes in bacterial families (Fig. 7) are significantly dependent on the treatment condition (P=0.001) being either control or stress, as opposed to the timepoint at which the samples were taken (Fig. 8a). The treatment-dependent microbial changes in the samples can be further demonstrated by showing the β-diversity among samples using an itemized analysis of how each bacterial order is influenced specifically by control or stress conditions (Fig. 8b), which provides additional detail not visualized by the ordination plots. Herein, the analysis of the relative abudance of bacterial families is recapitulated on the bacterial order level and paired with an analysis on inter-sample β-diversity, reiterating the significant effect of treatment condition on microbial composition between samples and showing that the day of CVS is of lesser relevance (Fig. 8b).

a)



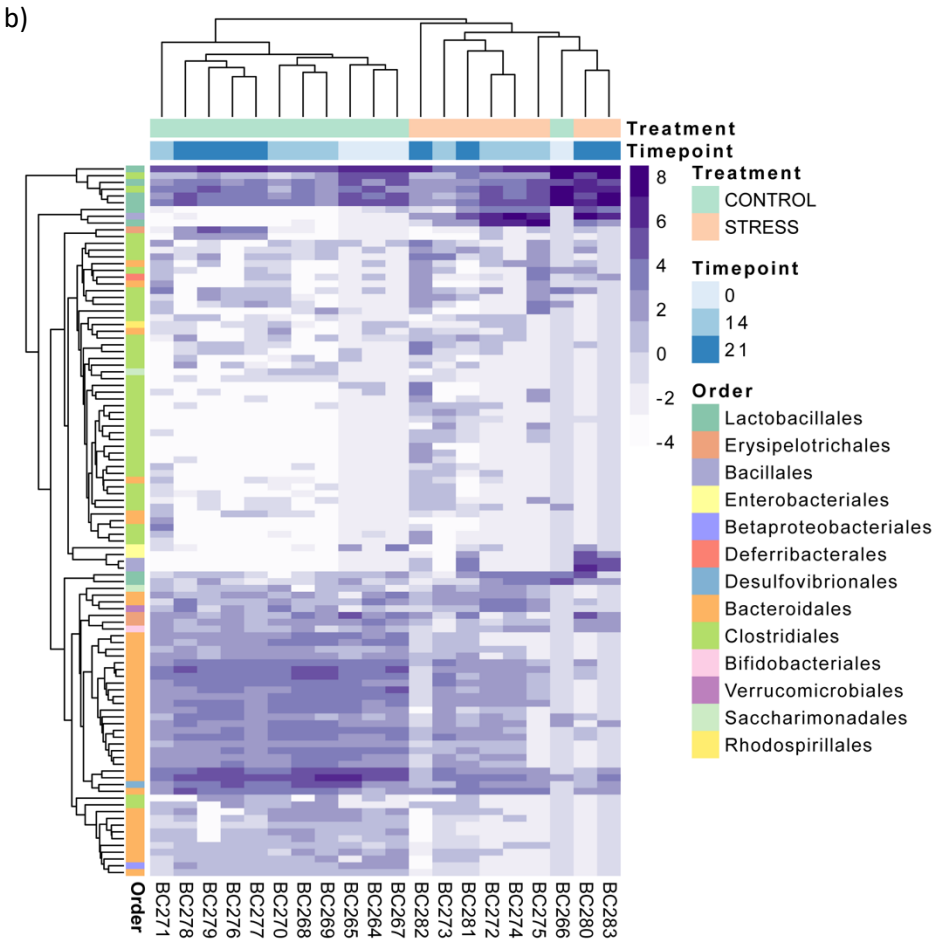


Figure 8 – PCA of control and stressed groups during CVS. (a) Aitchison ordination plots of four principal components showing the relatedness in species abundance between control and stressed samples across Day 0, Day 14, and Day 21. (b) Heat map showing heirarchal sample clustering across CVS timepoint and treatment condition as it relates to bacterial order abundance.

Human longitudinal studies and rodent models have shown that chronic stress decreases the α-diversity of the gut microbiome[118–120]. The α-diversity is a measure of the diversity within a community, and has two components: richness in the number of species, and evenness of the abundance of each species[121]. The Shannon and Simpson indices can be used to calculate the α-diversity within each sample of ileal contents[121]. The Simpson index, visualized in inverse, conveys the probability that two samples randomly drawn from the same community will be of

different species[121]. On days 14 and 21, the inverse Simpson index indicates that CVS increases the probability of selecting two microbes of the same species from the ileum of a stressed subject (Fig. 9a). The Shannon index indicates the level of uncertainty about the identity of a randomly chosen sample from the population, such that the presence of more species increases the uncertainty[121]. On days 14 and 21, the Shannon index indicates that under CVS, a randomly selected microbe taken from the ileum of a stressed subject can be identified with more certainty because of the decrease in the number of overall species (Fig. 9b). Overall, the inverse Simpson and Shannon indices suggest that CVS decreases community diversity in stressed mice as early as day 14 of stress (Fig. 9a, b).

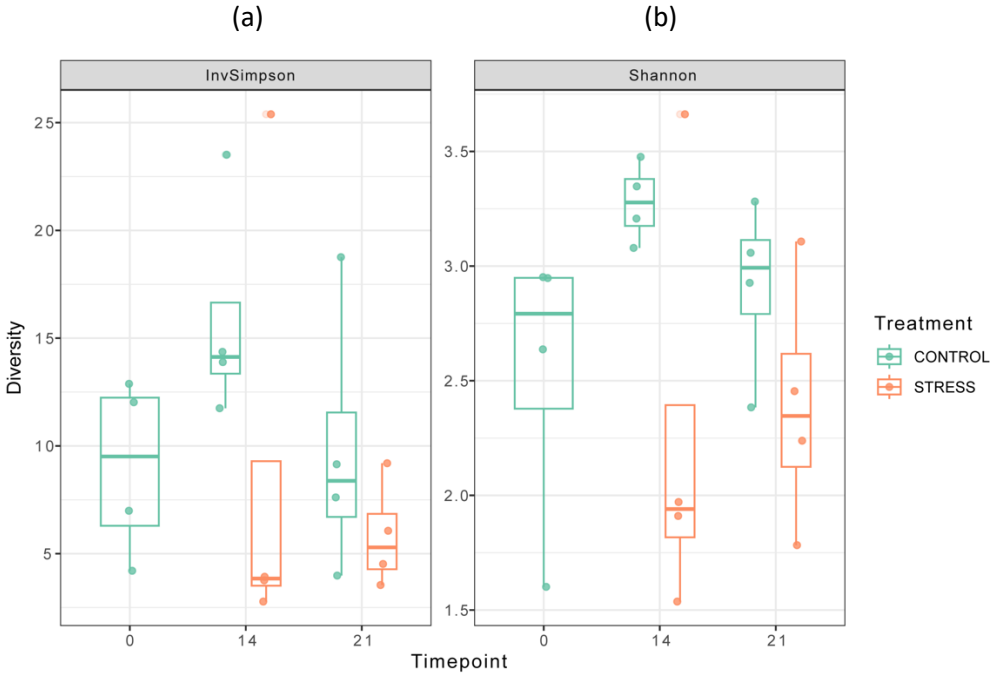


Figure 9 – Measurement of the α-diversity of the ileal bacterial communities in control and stressed mice on day 0, day 14, and day 21 of CVS. (a) Inverse Simpson's diversity index of control ($n = 4$) and stressed ($n = 4$) female mice. (b) Shannon diversity index of control ($n = 4$) and stressed ($n = 4$) female mice.

Intestinal Lipocalin-2 (LCN2) level in the lumen is increased under CVS

Lipocalin-2 (LCN2) in the gut reflects the activation of host nutritional immunity, a form of antimicrobial innate immunity that is stimulated by intestinal pathogens or perturbation of the microbiome[122–124]. In the gut, LCN2 is released by neutrophils, IECs, and macrophages to sequester iron, which is essential for microbe survival, thereby constraining bacterial growth through nutrient deprivation[122–124]. Quantitation of LCN2 content in stool by ELISA revealed that both male and female stressed mice have significantly higher levels of LCN2 than control mice on day 21 of CVS (P=0.0006) (Fig. 10). Moreover, the progressive increase of LCN2 release is specific to the CVS condition, as the control condition shows no significant change in LCN2 level between day 1 and day 21, while stressed mice show a significant upward trend (P=0.0404) (Fig. 10). These data indicate that under CVS, innate immune surveillance is triggered to deploy nutritional immunity.

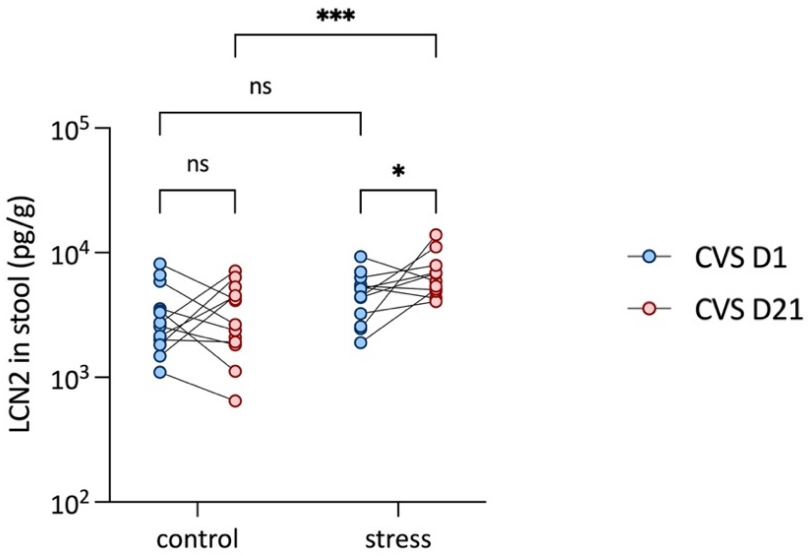
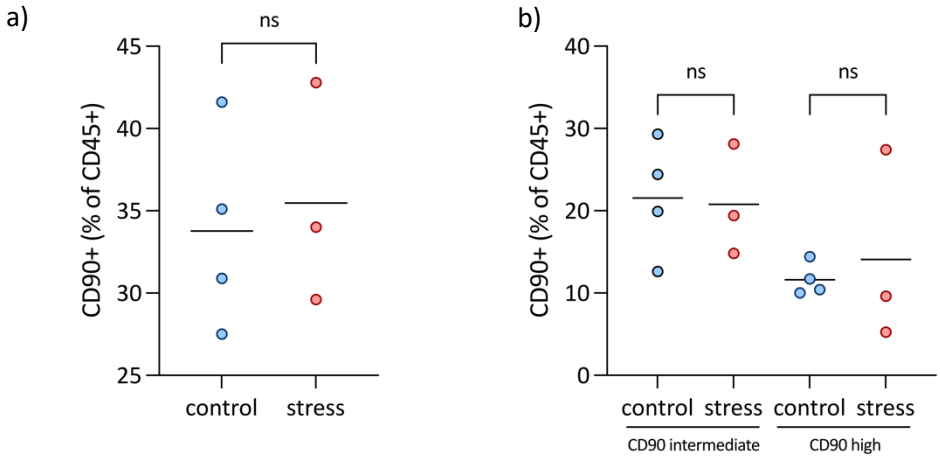


Figure 10 – Longitudinal comparison of stool LCN2 levels prior to CVS intervention and post final CVS intervention. LCN2 protein in stool was quantified by ELISA before initiation of CVS (D1) and at the end of CVS (D21) in control (*n*=16) and stressed (*n*=16) mice. Significance was determined by multiple comparisons using a mixed-effects model.
(ns P>0.05; *P≤0.05; **P≤0.01; ***P≤0.001).

CVS does not affect the proportions of leukocyte populations that interact with the gut microbiota

Chronic stress models in mice coincide with dysbiosis in the gut[106,109,118]. Hence, I chose to examine the effects of CVS on cellular immunity in the intestinal lamina propria in the context of host-microbe interactions. At the end of week three of CVS, stressed and control mice were sacrificed to harvest the ileum. The lamina propria mononuclear cells (LPMCs) of the ileum were isolated for evaluation by flow cytometry.

The first immune compartment of interest was the $CD90^+$ lymphocyte population (Supp. Fig. 1) which encompasses the cells responsible for IL22-mediated protection of the gut mucosal barrier. Namely, Th17 cells and type 3 innate lymphoid cells (ILC3), which are susceptible to GC-mediated apoptosis under acute stress[19] In a mouse model of acute stress, the attrition of the heterogeneous pool of $CD90^+$ cells was shown to be permissive to inflammatory dysbiosis and compromised intestinal barrier integrity[19]. Under the influence of CVS, however, control and stressed female and male mice showed no difference in the proportion of total $CD90^+$ cells (Fig. 11a, b). Further, control and stressed mice showed no difference in the proportions of $CD90^{intermediate}$ and $CD90^{high}$ within the total $CD90^+$ population (Fig. 11c).



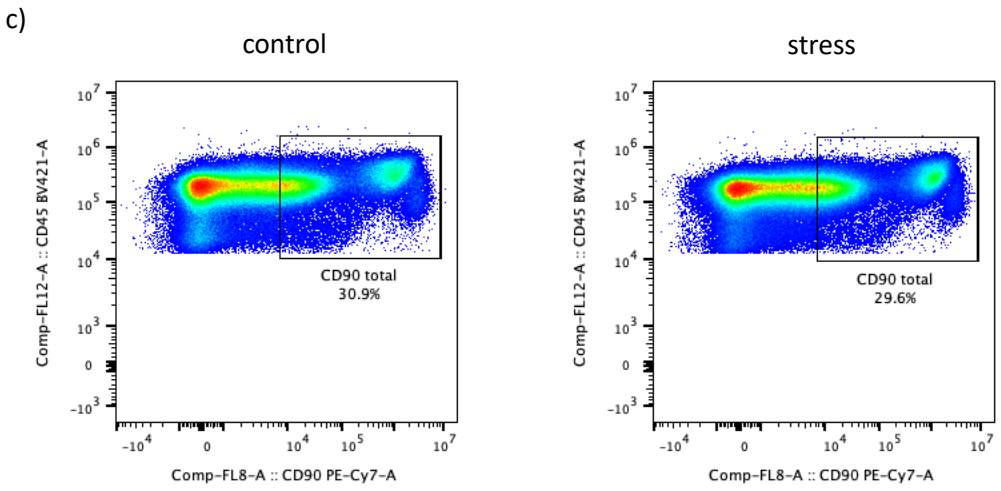


Figure 11 – Proportion of $CD45^{+}CD90^{+}$ cells in the ileum of control and stressed mice.
(a) Total $CD90^{+}$ cells as a percentage of total $CD45^{+}$ cells in control (*n*=4) and stressed (*n*=3) female mice. Significance was determined with a two-tailed Mann-Whitney test (ns P>0.05, no significance). (b) $CD90^{intermediate}$ and $CD90^{high}$ cells as a percentage of total $CD45^{+}$ cells in control and stressed mice. Significance was determined using a Kruskal-Wallis test with Dunn's multiple comparisons test (ns=no significance). (c) Representative flow cytometry of $CD45^{+}CD90^{+}$ cells in control and stressed female mice.

I next assessed the impact of CVS on the proportion of $CD4^+CD25^+Foxp3^+$ regulatory T cells (T_{reg}) in female mice. GCs are potent modulators of T_{reg} differentiation and function[125,126]. T_{reg} serve a tolerizing, homeostatic, and attenuating immune function which extends to tolerance to the microbiome[127]. Since CD is characterized by aberrant inflammation, it follows that disruption of the T_{reg} compartment may be involved in disease flares as is suggested in pre-clinical models of colitis[128,129]. Prescribed GC-based therapy can promote T_{reg} function as part of its anti-inflammatory medicinal effect[125], but humans experiencing an acute stressor show defective immune regulation marked by a decrease of circulating T_{regs} coinciding with an overexpression of GRs and β-adrenergic receptors[130]. These two findings allude to the potential for chronic stress to impair regulatory immunity by chronic exposure to GCs. I predicted that the $CD4^+CD25^+Foxp3^+$ T_{reg} compartment (Supp. Fig. 2) would be susceptible to either attrition or proliferation by CVS, impairing host tolerance of the microbiome. However, I found that CVS did not modify the proportion of T_{regs} in stressed mice as compared to control mice (Fig. 12a, b).

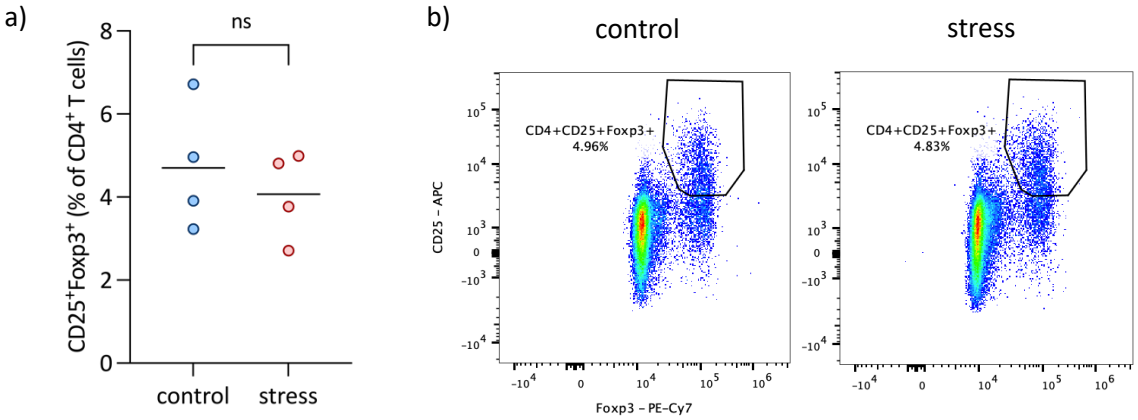


Figure 12 – Proportion of $CD25^+Foxp3^+$ regulatory T cells in the ileum of control and stressed mice. (a) $CD25^+Foxp3^+$ cells as a percentage of total $CD4^+$ cells in control (*n*=4) and stressed (*n*=4) mice. Significance was determined with a two-tailed Mann-Whitney test (ns P>0.05, no significance). (b) Representative flow cytometry of $CD25^+Foxp3^+$ regulatory T cells.

CVS skews the immune transcriptional profile toward inflammation and Th17 activation

Despite the unchanging frequency of the selected immune populations, the transcriptional profile of the ileal lamina propria was assessed to reveal possible functional deficits unrelated to cell quantity. I probed the transcriptional profile of the terminal ileum in control and CVS-exposed mice using reverse transcription quantitative polymerase chain reaction (RT-qPCR). This analysis revealed that CVS elicited a significant increase in the expression of *Il1β*, *Il6*, *Il23*, *Il22*, and *Il17a* (Fig. 13).

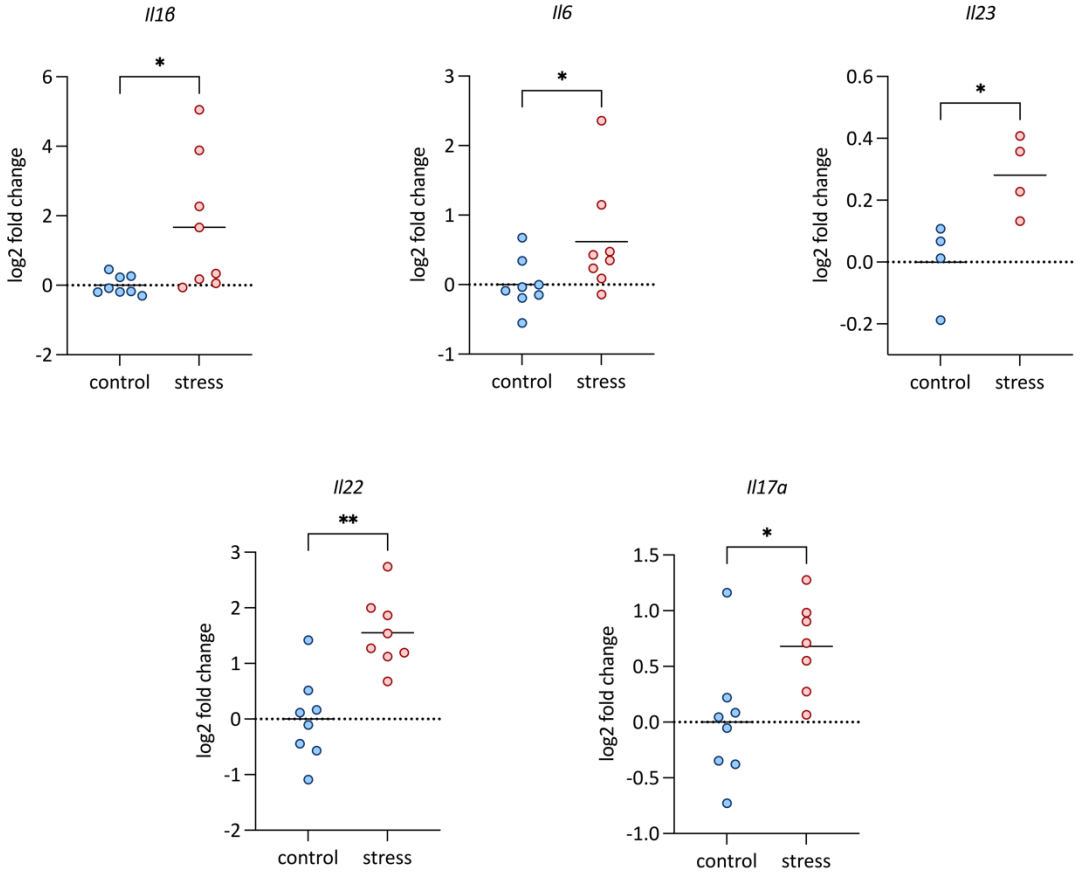


Figure 13 – Gene expression of cytokines in the terminal ileum. Gene expression in the ileum was assessed with RT-qPCR and compared between control (n = 8) and stressed (n = 8) male mice. Significance was determined using a two-tailed Mann-Whitney test. (ns P>0.05; * P≤0.05; ** P≤0.01; *** P≤0.001).

CVS does not facilitate AIEC colonization in the gut

Immunomodulation by acute stress has been previously found to permit a profound bloom of CD-associated AIEC in the gut lumen and tissue[19]. The observations of the effects of acute stress bring to question whether CVS similarly facilitates a disease-state microbiome. Hence, I applied the CVS model to an established AIEC colonization model[19]. Two groups of female C57BL/6N mice were colonized with AIEC, and one group was subjected to CVS while the control group was left undisturbed. Upon infection, AIEC colonization in the intestine was left to stabilize for five days. On day 5 post-infection, the CVS protocol was initiated. Consistent with the naïve model, CVS did not result in weight stagnation or weight loss in the presence of AIEC in female mice (Fig. 14).

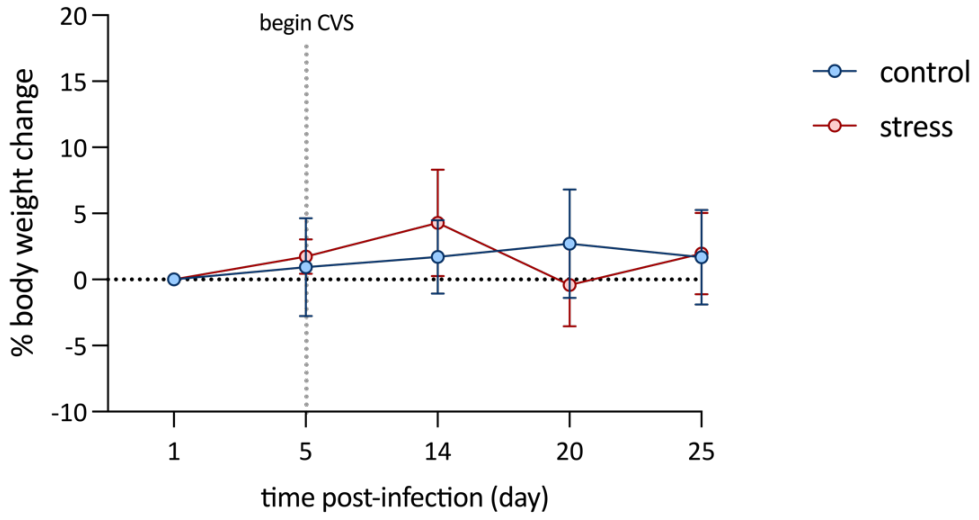


Figure 14 – Percent body weight change throughout AIEC colonization and CVS. Female control (*n*=4) and stress (*n*=4) groups. Error bars represent standard deviation.

I collected stool samples at the same time daily to quantify the luminal AIEC burden in control and stressed groups. Female stressed mice did not show differential persistence of AIEC as compared to control groups. After beginning CVS, all mice experienced an oscillation in AIEC burden in the stool ranging between 10^7 CFU/g stool to levels below the limit of detection (Fig. 15).

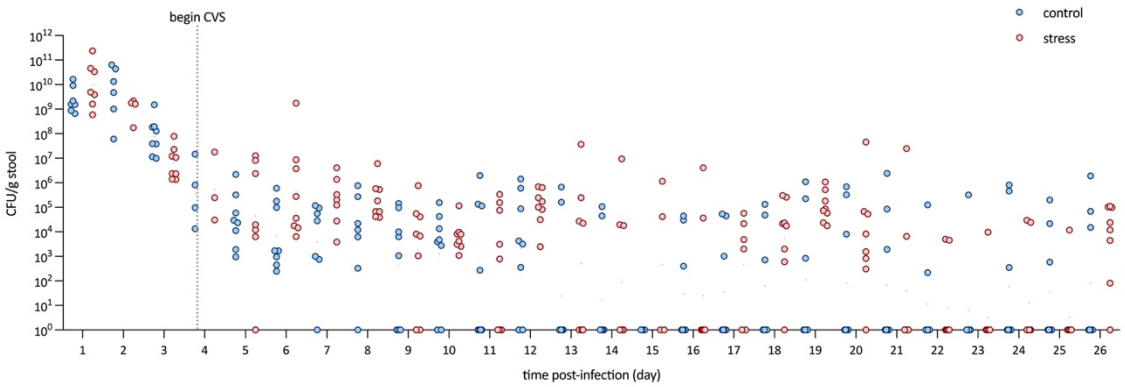


Figure 15 – AIEC colonization under CVS. AIEC burden in stool collected from CVS (n = 8) and control (n = 8) female mice.

Further, in line with our previous study on the apoptotic effects of acute stress on the $CD90^+$ leukocyte population[19], I quantified $CD90^+ROR\gamma^+$ cells in female mice to assess whether CVS exerts the same effects as acute stress in combination with our established AIEC colonization model. However, I found that CVS did not change the proportion of $CD90^+ROR\gamma^+$ cells in stressed, AIEC-colonized female mice (Fig. 16a, b).

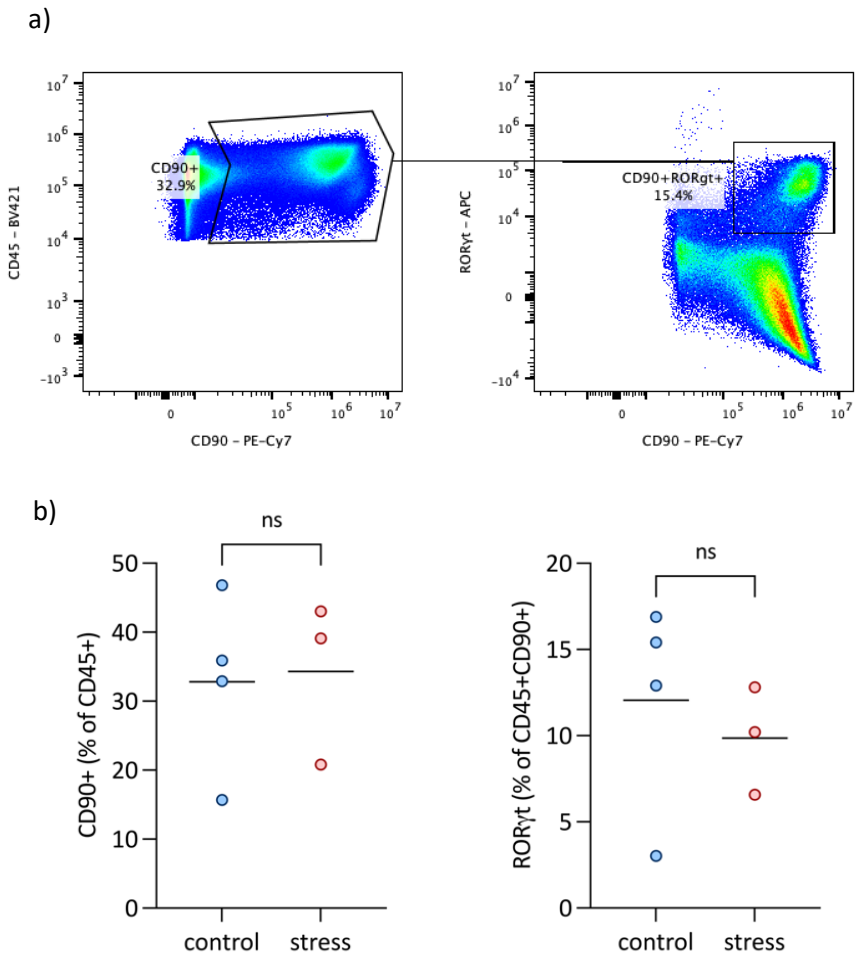


Figure 16 – Proportion of CD45$^+$CD90$^+$RORγt$^+$ cells in the ileum of AIEC-colonized control and CVS mice. (a) Representative flow cytometry plots of CD45$^+$CD90$^+$RORgt$^+$ cells. (b) Proportion CD45$^+$CD90$^+$ and CD45$^+$CD90$^+$RORgt$^+$ in control (n = 4) and stressed (n = 3) mice. Significance was determined with a two-tailed Mann-Whitney test (ns P>0.05, no significance).

I sought to quantify the changes in stool LCN2 content over the course of CVS in AIEC-colonized mice. I collected stool on days 1, 7, 14, and 21 of CVS to capture a full timeline of potential perturbations in LCN2 levels. In contrast to stressed naïve mice, AIEC-colonized mice subjected to CVS did not show any notable change in LCN2 content in the stool over the full course of CVS (Fig. 17).

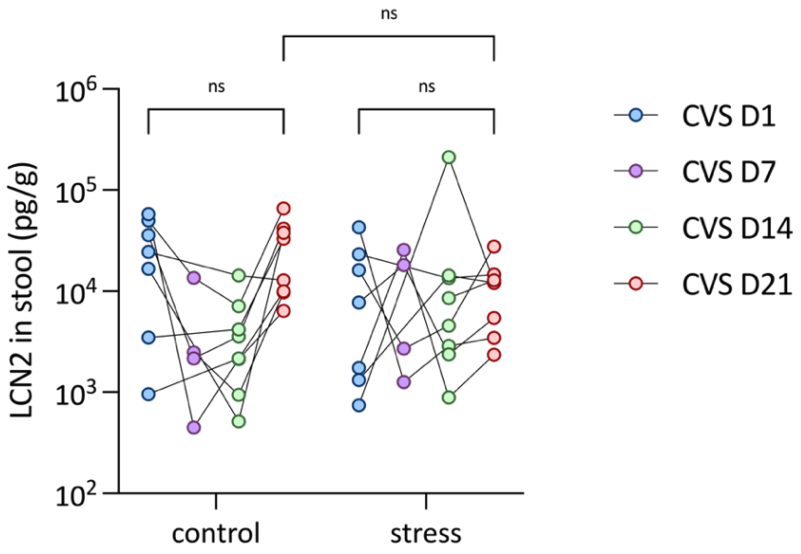


Figure 17 – Longitudinal comparison of stool LCN2 levels in AIEC-colonized mice prior, during, and post CVS intervention. LCN2 protein in stool was quantified by ELISA before initiation (D1), during (D7, D14), and at the end of CVS (D21) in control (*n*=8) and stressed (*n*=8) mice. Significance was determined by multiple comparisons using a mixed-effects model (ns P>0.05, no significance).

Repeated restraint stress presents a viable alternative for a mouse model of chronic stress

In light of the limitations of CVS, including possible microbiome contamination and the impractical experimental protocol, I aimed to test the viability of a more efficient chronic stress model, repeated restraint stress (RRS) (Fig. 3). I colonized a group of female mice with AIEC (day 0) and on day 5 post-infection, I began implementing RRS, wherein mice were restrained once a day for 3 hours over the course of 7 days, and sacrificed after 8 days . Over the course of RRS, the stressed group experienced significant weight loss in comparison to the control group. The mean weight loss of the stressed group was -7.82% and the control group -2.57% of the body weight on Day 1 of RRS (P=0.0015) (Fig. 18).

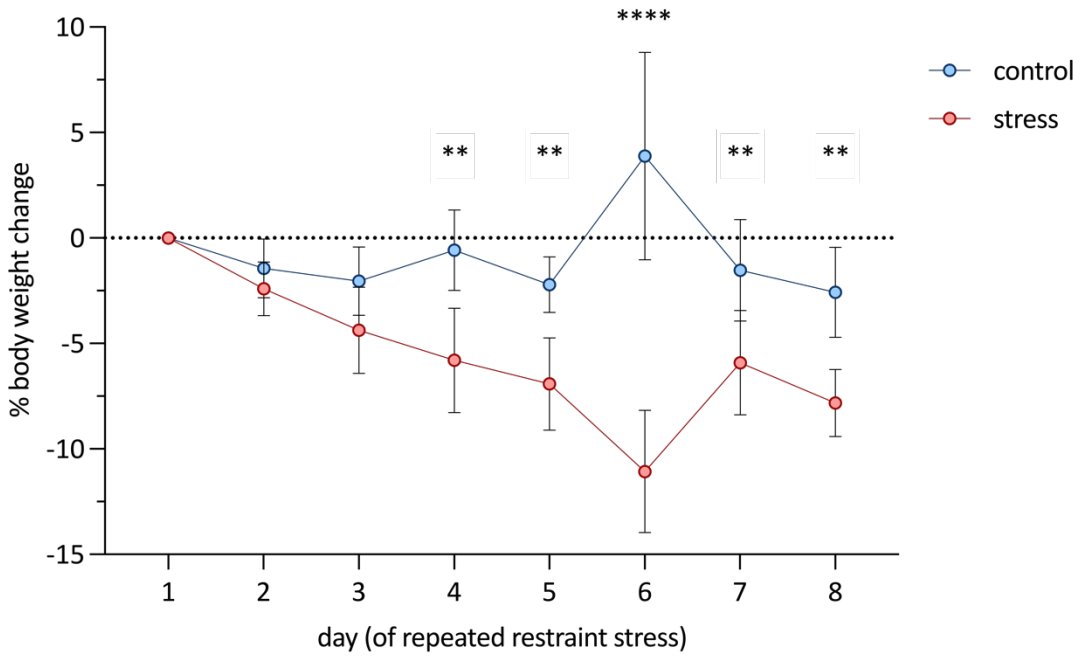
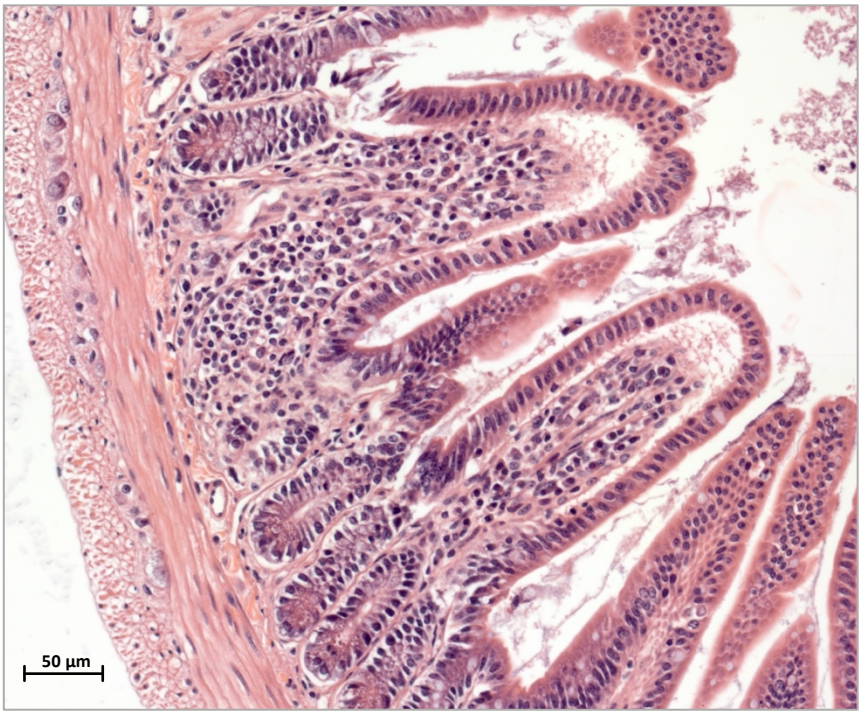


Figure 18 – Percent body weight change throughout repeated restraint stress. Female control (*n*=4) and stress (*n*=4). Error bars represent standard deviation. Significance was determined with a two-way ANOVA with the Geisser-Greenhouse correction and Šídák's multiple comparisons test. (ns P>0.05; * P≤0.05; ** P≤0.01; *** P≤0.001).

I also assessed the effect of RRS on the structural integrity of the terminal ileum through a cross-sectional histopathological evaluation. The control and stressed groups both exhibited a mildly compromised mucosa as marked by epithelial barrier erosion on the villi (Fig. 19a). Further, both groups exhibited mild-to-moderate infiltration of mononuclear leukocytes into the epithelial layer, and an increase in polymorphonuclear leukocytes in both the lamina propria and the epithelium (Fig. 19a). The combined histological score indicates that both control and stressed groups show a similar degree of damage to the ileal barrier, where the stressed group trends toward a higher histopathological score (Fig. 19b).

a) **CONTROL**



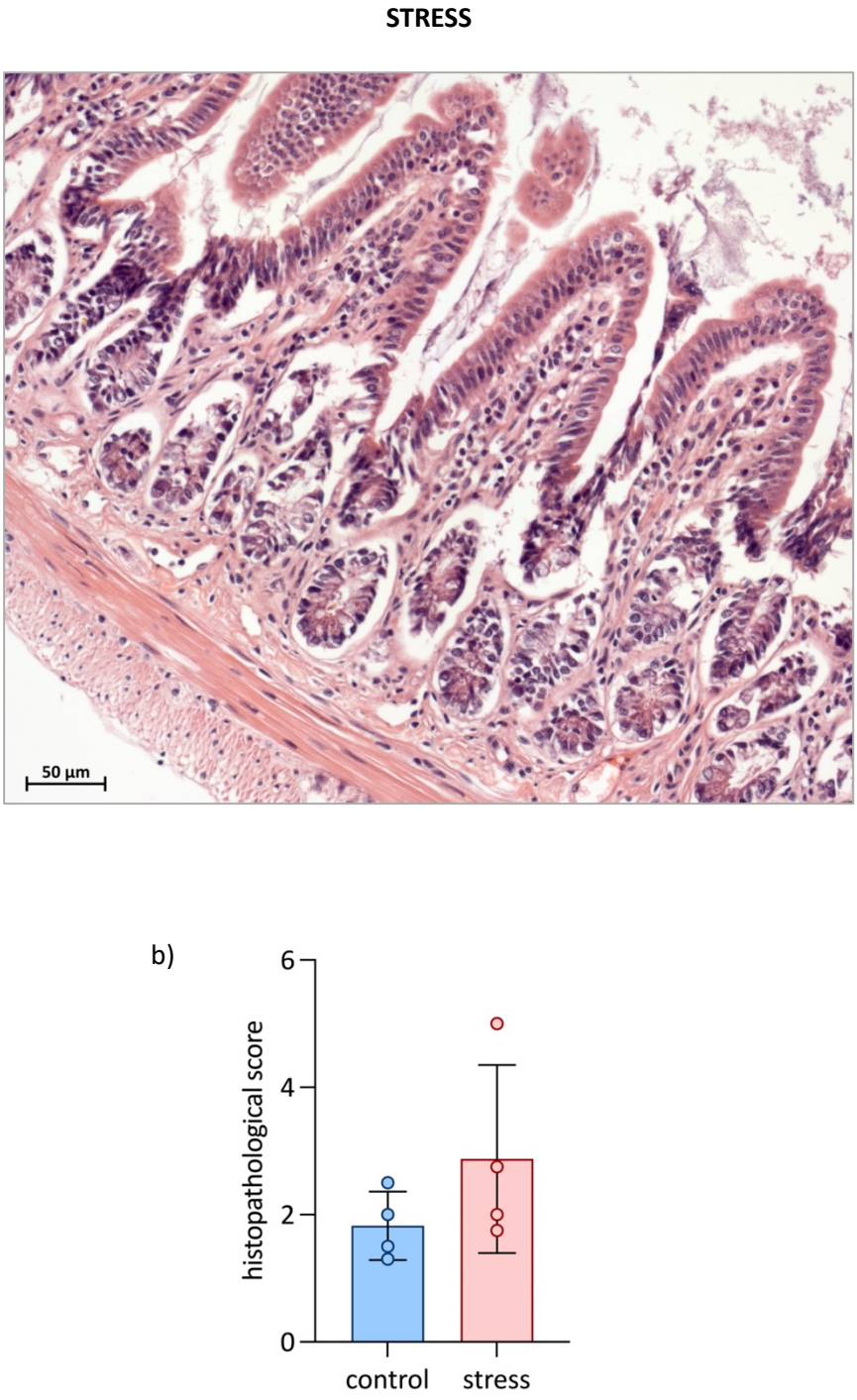


Figure 19 – Histopathological evaluation of intestinal tissue from control and RRS mice. (a) Representative H&E stained cross sections of the ileum in control (left) and stressed (right) mice. (b) Histopathological scoring of the ileum in control (n = 4) and stressed (n = 4) mice. Points represent individual mice. Error bars represent standard deviation.

I quantified the luminal burden of AIEC over the course of RRS by collecting stool at 12:00h daily before starting restraint stress. Between day 1 and day 12 post-infection, the control and stressed groups did not show differential persistence of AIEC (Fig. 20). Both groups maintained levels around a mean of 10^6 CFU/g stool. In addition, neither of the groups showed tissue invasion by AIEC, as indicated by the absence of detectable AIEC in harvested ileum, cecum, and colon sections (data not shown).

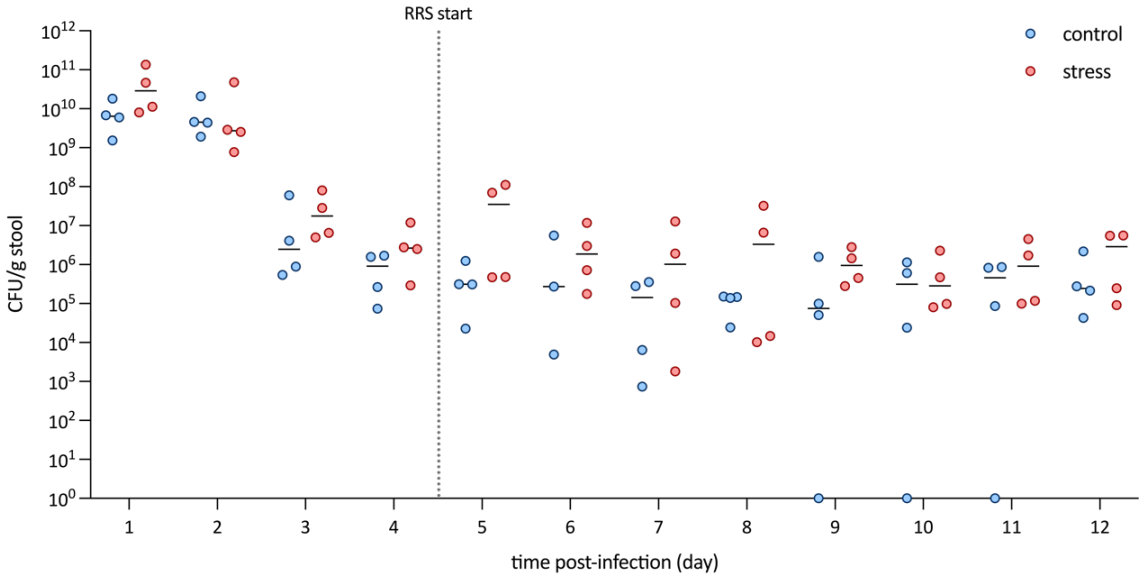


Figure 20 – AIEC colonization under RRS. AIEC burden in stool collected from RRS (*n*=4) and control (*n*=4) mice.

DISCUSSION AND FUTURE DIRECTIONS

Chronic psychological stress is emerging as an important disease modifier for CD. In CD patients, high perceived stress is a major predictor of the reactivation of clinical inflammation after a period of disease quiescence[35–37]. The association between stress and intestinal disease has been closely scrutinized for decades using rodent models of stress, uncovering intestinal barrier defects and, most recently, microbial perturbation[106,109,118]. However, there remains a disconnect between observations of stress-induced immunomodulation and coinciding changes in the microbiome. With the knowledge that both stress and the microbiota are cornerstones of CD pathophysiology, my thesis aimed to better understand how stress impacts the microbiota in a mouse model of AIEC colonization[19]. The pathophysiology of chronic stress as it relates to the microbiota is now focused on discerning disease-relevant changes within microbial taxa, the cause and effect of such microbial perturbations, and uncovering the full scope of immuno-modulation in the gut. Thus, **I hypothesized** that chronic stress is a maladaptive modulator of intestinal mucosal immunity, creating a gut environment that is permissive for disease-state dysbiosis.

The hypothesis of this study is predicated on the profound immunomodulatory capabilities of GCs across homeostatic, therapeutic, and stress conditions[54]. At the end of the CVS protocol (day 21), the level of corticosterone in the plasma of male and female mice was quantified using LC/MS with the expectation that CVS would increase levels of corticosterone, similarly to how chronically stressed humans experience an increase in cortisol. I observed a sex-dependent difference in the effects of CVS, wherein male mice were more susceptible to stress-dependent increase in circulating corticosterone, and female mice showed highly variable but unremarkable changes (Fig. 6b). Moreover, female mice possessed overall higher baseline levels of corticosterone than male mice (Fig. 6b). It is worth noting that corticosterone levels were only

evaluated on day 21 of CVS, which is the experimental endpoint. It is unclear whether the sex difference persists through the duration of CVS, and the timepoint at which the increase in corticosterone occurs in males. The field of chronic stress in the physiological context is only recently recognizing sex as an important variable. It is known that female rodents have higher baseline levels of corticosterone than males[131,132], which is in line with the data presented in this study. However, current studies remain conflicted on whether males or females are more sensitive to stress-induced increases in corticosterone[131–133]. In both cases, it appears that estradiol and testosterone are major regulators of the HPA axis, capable of both stimulating and attenuating the stress response through several mechanisms which are comprehensively reviewed by Oyola and Handa[131]. The use of different stress models, environmental conditions, rodent strains[134], and even the sex of the researcher that is handling the mice[135] are all confounding variables which must be considered when retroactively drawing comparisons between sexes in past studies. The possible sex-difference in the corticosterone response under CVS should be kept in mind while interpreting the findings of this work.

Models of acute and chronic stress are known to compromise the intestinal barrier within the ileum and colon, as indicated by histological observations of epithelial erosion and leukocyte infiltration[19,106,109]. It then follows that the insult to the gut barrier is accompanied by significant weight loss[115]. Regardless of sex, mice in the current study did not experience weight loss or stagnation throughout CVS (Fig. 4). Moreover, histological analysis showed that CVS did not cause visible damage to the intestinal mucosa as indicated by an intact and non-inflamed barrier in the ileum and colon of stressed and control mice (Fig. 5). However, this does not exclude the possibility of ultrastructural defects in the barrier as previously reported[105], which are not seen in histological analysis, but rather by transmission electron microscopy. The majority of studies which report barrier damage use a two hit model, wherein the chronic stress is combined with

another insult such as DSS[109], bacterial infection[136], or genetic deficiency[111]. Though there are a few studies that have shown barrier damage as an outcome of chronic stress alone[104,105], many other studies are in line with my findings, showing that chronic stress alone has an indolent disease-priming effect as opposed to being a solitary pathological agent. I aimed to characterize how chronic stress alone can increase intestinal vulnerability to disease by describing its sole histological, transcriptomic, and tissue-level effects. However, the next step is to observe these hypothesized vulnerabilities in a more relevant context by pairing CVS with established models of colitis or CD risk factors. I propose that CVS should be applied to IL10 knockout (IL10KO) mice, which is a well-established model in our lab. The absence of regulatory IL10 in the IL10KO model predisposes the mice to uncontrolled inflammation which spurs spontaneous colitis throughout the intestinal tract. Previous work suggests that IL10KO mice subjected to CVS may be a feasible model for stress-induced spontaneous colitis[111], which can be evaluated through histological analysis. Moreover, as opposed to models of chemical colitis which primarily affect the colon, IL10KO mice allow for the study of CD within the ileum which is the primary disease site in humans.

Models of chronic stress in rodents have demonstrated intestinal dysbiosis for which the cause and ramifications remain unknown[106,109,118]. I sought to evaluate the effect of CVS on microbial composition and diversity within the ileum, in addition to capturing a temporal view of these changes by obtaining the ileal contents from female mouse cohorts on day 0, day 14, and day 21 of CVS and submitting these samples for 16s ribosomal RNA (rRNA) sequencing. These data showed that CVS generally led to a significant contraction of Muribaculaceae and Lactobacillaceae persisting through days 14 and 21, concomitant with the expansion of Staphylococcaceae which was also maintained between days 14 and 21 with moderate contraction at the latter timepoint (Fig. 7). The stressed group also showed a transient bloom of

Aerococcaceae at day 14 which diminished by day 21. Control groups steadily maintained dominance of Muribaculaceae and Lactobacillaceae with no detectable Staphylococcaceae or Aerococcaceae at any time point. The blooms of Staphylococcaceae and Aerococcaceae in the stressed group are unclear in their origins and implications for the rest of the microbiota as well as host physiological responses. It is possible that these families were present at low frequency in the native microbiome of the mice, and CVS indeed resulted in an unknown immune deficit which permitted their expansion. Another possibility is that these families were not native to the stressed group microbiome, but were introduced as contamination during the course of CVS as a result of frequent mouse handling, which would be a major limitation of the CVS model.

The contraction of the Muribaculaceae family under CVS contradicts previous findings within a chronic social stress model which found that this family expanded and dominated the microbiome[106]. However, in the current study, the introduction of Staphylococcaceae into the stressed group may be skewing the true effects of chronic stress on the microbiota, and thus the decrease in Muribaculaceae and Lactobacillaceae under CVS may be an artifact of the competitive colonization by Staphylococcaceae. Cage effects may also be confounding the results as this study was not longitudinal, but each time point was a different cohort of mice and all CVS cages were handled together while controls remained secluded. Possible confounding variables notwithstanding, the introduction of Staphylococcaceae presents a few potential findings. The first being that models of intestinal colonization by members of the Staphylococcus family often require the pre-administration of antibiotics to facilitate colonization[137,138], suggesting that the mice in the current study may have experienced stress-induced dysbiosis which permitted colonization by Staphylococcaceae. Another interesting observation is that on day 21, the stressed mouse which showed the most control over Staphylococcaceae colonization experienced a concomitant bloom of the Lachnospiraceae family which was not observed in any other mice

(Fig. 7). Many genera within the Lachnospiraceae family are butyrate-producing commensals which boost intestinal immunity, microbiome homeostasis, and barrier integrity. It is interesting to postulate that increased Lachnospiraceae was advantageous toward restoring microbial homeostasis. Lastly, the Shannon and inverse Simpson indices revealed that CVS decreases the overall diversity in the ileal microbiome of stressed mice (Fig. 8). This is consistent with previous observations in the study of stress[106,109], and is a common deficit of the microbiome in CD patients[1]. However, these data require more biological replicates to more clearly demonstrate the differences in diversity.

Thus far, the study of stress and its effects on the microbiome has been limited to several variables, including sex, the rodent microbiome, and specific intestinal regions of interest. Many of the recent studies focus solely on male rodents[109,118,119], which limits the translational potential of the findings. In addition, the effects of chronic stress in CD patients are difficult to extrapolate from the SPF microbiome in mice, since it is markedly different from the human microbiome. I propose that in the study of stress as it relates to the intestinal microbiome, humanized microbiota should be used in mice to more closely recapitulate microbial changes as they would occur in the human GI tract and generate more translatable observations. Generally, human studies of stress and its effects on the gut microbiome are limited by the invasiveness of tissue analysis, the subjectivity of perceived stress, and the abundance of confounding variables encountered in daily life. Nikolova and colleagues recently published a meta-analysis on the known microbial changes in the stool of adults with psychiatric disorders[139]. The findings included changes in taxa, diversity, and the metabolome. Individuals with bipolar disorder showed a marked decrease in the number of bacterial species present within their stool samples in comparison to matched controls, which conveys a decrease in α-diversity[139]. Moreover, these patients showed a reduction in butyrate-producing bacteria, including the *Faecalibacteria* genus within the Ruminococcacea

family and the *Coprococcus* genus within the Lachnospiraceae family[139]. These results are in line with my observations of naïve mice under CVS. However, the main concern is the maladaptive microbial effect of chronic stress on individuals with CD, which would necessitate a human study on a large cohort of individuals with CD which are classified by perceived stress scores prior to sequencing of the microbiota in stool samples. This could uncover trends in the gut microbiome that underpin CD flares and inform targeted therapies to help patients overcome stress-induced inflammation. Lastly, the biogeographic region of interest is a limitation of both human and rodent studies. The human GI tract is not accessible without invasive procedures. On the other hand, the rodent studies on the microbial etiology of CD and the maladaptive effects of stress on the microbiota are largely limited to stool samples or the lumen of the colon. The ileum is a largely understudied region of the gut, especially at the intersection of CD and stress, and thus my work serves to address this gap in the knowledge on the most common site of inflammation in CD[2,116,117].

The quantification of LCN2 protein content in the stool can be used as a broad indicator of the disruption of host-microbe homeostasis. Upon stimulation of innate immune surveillance by microbial dysbiosis or acute infection, LCN2 is released by neutrophils, macrophages, and IECs as a form of nutritional immunity[122–124]. Herein, LCN2 binds bacterial siderophores carrying iron, an essential metal, thereby constraining bacterial growth through nutrient deprivation[122–124]. I found that stressed mice had significantly higher LCN2 content in stool than control mice on Day 21 of CVS, and that the significant increase from Day 1 to Day 21 was only present in the stressed group (Fig. 10). In line with my hypothesis, these data suggest that CVS may be precipitating microbial perturbation, or another antagonizing signal, which elicited the increase in LCN2 release.

It is difficult to ascertain the basis for the increase in LCN2, and whether it is sufficient to increase gut susceptibility to inflammation or if it is buffered by homeostatic immune processes. As previously mentioned, the composition of the ileal microbiome does not present any obvious patterns to which I can attribute the increase in LCN2 under CVS. The bloom of Staphylococcaceae in the stressed mice in Day 14 and Day 21 may be the culprit, but this is not a reliable hypothesis as it is not clear whether Staphylococcaceae were introduced across all the experiments showing elevated LCN2. Moreover, the origin of LCN2 is uncertain. Neutrophils are the principal source of LCN2 in response to dysbiosis or infection in the gut[123]. However, histological analysis showed no infiltration of neutrophils into the lamina propria or the epithelium (Fig. 5). Tissue-resident macrophages and IECs are secondary sources of LCN2 which are constitutively present in the gut[122,124], and thus may be responding to a stimulus induced by CVS in the gut lumen. The determinants of the source of LCN2 are unknown, and it would be interesting to examine which luminal signals elicit LCN2 release from different compartments. I propose the use of immunofluorescent imaging using markers for IECs, tissue-resident macrophages, and LCN2 to discern the possible differential production of LCN2 within these cells in stressed mice. Moreover, the content of LCN2 in the stool is a global indicator of average levels throughout the entire GI tract and cannot be assigned to a specific geographical region of the gut. The increase in LCN2 under CVS may be a consequence of microbial perturbations within the cecum or colon, which can also be ascertained using immunofluorescent imaging of each region of the GI tract.

Lastly, the elevation of LCN2 levels in stool under CVS can be closely related to humans. It has been posited that chronic stress in healthy individuals elicits low-grade inflammation which precipitates elusive stress-induced GI symptoms[140]. More crucially, individuals with CD show an increase in stool calprotectin, a similar form of nutritional immunity which is indicative of gut

inflammation and dysbiosis[141]. Unraveling the trigger, source, and implications of LCN2 elevation in stressed mice holds significant potential for translatable knowledge on the effects of chronic stress in the human GI tract. To better study the effects of innate immune activation under chronic stress, I suggest the application of CVS to IL10KO mice, which may amplify the LCN2 phenotype in a manner similar to the increased vulnerability of CD patients.

To interrogate the effects of CVS on intestinal immunity more rigorously, I examined the post-CVS cellular landscape with flow cytometry. The immunophenotyping rationale was based in selecting leukocyte populations that are involved in host interaction with the gut microbiota. Thus, I aimed to quantify ILC3/Th17 cells which are responsible for IL-22-mediated barrier protection[142]; T_{regs} which partly mediate tolerance of the microbiota[128]; and plasma cells which release microbe-binding IgA[143]. The ILC3/Th17 population was demarcated as $CD45^+CD90^+$ after gating for populations which were not of interest (Supp. Fig. 1). I found that CVS does not change the proportion of $CD90^+$ cells among leukocytes in the ileum of male mice (Fig. 11), which is in contrast to the known effects of acute stress wherein the flood of GCs results in the apoptosis of $CD90^+$ cells, thereby compromising IL-22-mediated protection of the intestinal barrier and permitting colonization by AIEC[19]. This observation was also confirmed in female mice (data not shown). Similarly, CVS had no effect on T_{reg} populations in female mice (Fig. 12), though this has not been confirmed in male mice which appear to be more sensitive to GC increase by stress, and have been found to experience a durable increase in splenic $CD4^+CD25^+$ T_{reg} and serum IL-10, one of the regulatory effectors of T_{regs}[118]. That said, *Il10* gene expression in stressed male mice is comparable to that of control mice, suggesting that IL-10-mediated regulatory immunity is unaffected by CVS in male mice. I have not been able to examine IgA^+ plasma cells thus far, however plasma cell perturbation is less relevant to microbial control under stress[73,93]. The following transcriptional data, however, shift the focus from lymphoid to myeloid lineage cells as

a new population of interest and suggests functional changes within $CD90^+$ cells that are unrelated to cell quantity.

In transcriptional analysis, I found that the CVS model skewed gene expression within the terminal ileum toward a pro-inflammatory cytokine milieu. The classical inflammatory cytokines *Il1β* and *Il6* were significantly upregulated, along with a general shift toward the activation of Th17 immunity as marked by the upregulated *Il23/Il22/Il17a* signaling axis (Fig. 13). IL-6 is a canonical marker of inflammation, and, along with *Il1β*, has been long observed to be elevated in humans and rodents under stress as a form of subclinical chronic inflammation[63,144]. However, IL-6 is released from many sources including lymphocytes, myeloid cells, and several types of stromal cells, making it difficult to interpret this observation beyond the notion of general inflammation. The *Il1β* gene is primarily expressed by myeloid lineage leukocytes in response to a pattern recognition receptor (PRR) stimulation by pathogen- and danger-associated molecular patterns (PAMPs and DAMPs), driving acute inflammatory responses to facilitate defense against infection and injury. In the context of bacterial dysbiosis, it can be reasoned that the expansion of certain bacterial taxa causes an increase in stimulation of PRRs by their respective ligands; this is also in line with the observed increase in LCN2. For example, encroachment of Gram-negative bacteria, such as Enterobacteriaceae, would activate toll-like receptor (TLR) 4 signaling through increased lipopolysaccharide (LPS). Similarly, an increase in flagellated bacteria would signal through TLR5, or TLR2 would be stimulated by the lipoteichoic acid of Gram-positive bacteria[145]. However, it cannot be concluded that innate immune detection of the microbiota is the origin of the *Il1β* upregulation. As previously mentioned, 16s rRNA profile of the ileal microbiota does not point to any obvious trends of microbial triggers for *Il1β*; the colonization by *Staphylococcus* in stressed mice may potentially be the stimulus, but it is not certain to have been introduced in the specific pathogen-free (SPF) microbiome across all experiments. An easy method to investigate

the origin of *Il1β* upregulation would be to administer TLR antagonists during CVS, which should abrogate *Il1β* involvement. This is complicated by selecting which TLR to inhibit and the potential for the involvement of multiple TLR signaling cascades which converge at the master cytokine transcription factor NF-kB.

Most of all, it is not clear if the increase in *Il1β* transcription under CVS equates to functional Il-1β cytokine effects. *Il1β* is initially translated into an inactive pro-form which accumulates in the cytoplasm until activation by the inflammasome protein complex, whose assembly is also promoted by NF-kB[146]. Caspase-1, a protease from the inflammasome, cleaves pro-IL-1β into its active form[146]. To assess the functional potential of increased *Il1β* expression under CVS, a Western blot comparing the presence of pro-IL-1β and mature IL-1β should be conducted on protein isolates from ileal samples. Should there be an increase in mature IL-1β in the CVS condition, this would indicate that CVS provokes a pro-inflammatory state. Moreover, monocytes and tissue-resident macrophages are the main sources of *Il1β*[146], and these cells are highly susceptible to immunomodulation in the gut by both GCs and catecholamines[50,111]. Though histology has not indicated the infiltration of mononuclear cells into the gut epithelium, existing cells in the lamina propria can be differently polarized under CVS. Thus, it would be pertinent to evaluate the monocyte and macrophage populations under CVS with flow cytometry, probing for different types of these cells within the population.

The significant increase in the expression of cytokines along the IL-23/IL-22/IL-17A axis suggests that CVS skews the immune profile in the ileum toward a Th17 response, which can be either protective or inflammatory. The differentiation and maintenance of Th17 cells is driven by IL-1β, IL-6, and IL-23 produced by dendritic cells, macrophages, and IECs[147], which are all upregulated in the current study (Fig. 13). Cell populations that show Th17 traits are marked by the expression of RORγt (*Rorc*) , IL-23 receptor (*Il23r*), *Il17a*, *Il17f*, *Il22*, *Il6*, *Il21, Csf2*, *Tnf*, CC-

chemokine ligand 20 (*Ccl20*), and IL-1 receptor type 1 (*Il1r1*)[147]. These cells include NK cells, γδ T cells, and ILC3s located at secondary immune sites all over the body[147]. Preliminary data show that the expression of *Csf2*, *Il17f*, and *Tnf* were unchanged under stress (data not shown). IL-21 is a critical autocrine growth factor for Th17 cells and thus its expression should be evaluated to further probe the possibility of changes in cell proliferation[148]. As previously mentioned, I quantified the proportion of $CD90^+$ cells, which is a broad marker for Th17 and ILC3s as well as other cell types. I also quantified $CD90^+Ror\gamma t^+$ cells in stressed AIEC-colonized mice to increase specificity. In both cases, I found no change in cell populations. This leaves at least two possible scenarios: (1) the observed skew toward a Th17 response is based in functional altering of the existing Th17 population, rather than change in cell quantity; or (2) there are changes in a subpopulation of Th17 cells which require more refined gating to be discerned, as opposed to broad immunophenotyping by CD90 positivity. In any case, CVS shows a significant activation of Th17 signaling and exploring the downstream implications of this response should be prioritized in follow up work.

Th17 cells and ILC3s are common residents in the gut under normal conditions[149]. The role of IL-17 in the gut is mainly protective, where it directly stimulates IECs to produce tight junction proteins which reinforce and maintain integrity of the intestinal barrier[150]. Stimulation by IL-23 also causes ILC3s to release IL-22, another protective cytokine which acts on IECs to promote healing of the mucosal barrier and release of antimicrobial peptides in murine DSS colitis[151] or bacterial infection[152]. However, IL-17A and IL-22 both represent a double-edged sword as they have been found to play a pathogenic role in IBD under uncontrolled IL-23 signaling. The Th17 pathway was first implicated in a pathogenic role in IBD in 2003, wherein a comparative study found a significant increase in $IL\text{-}17^+$ cells and *Il17* expression in the colonic mucosa of CD patients in comparison to healthy individuals[153]. *Il23r* has also been discovered as a

CD susceptibility locus[154], and IL-22 can induce IL-18 in IECs as part of its barrier protective response to infection[155], which may account for the paradoxical role of IL-22 in IBD[156]. These findings have underpinned the approval of the anti-IL23 biologic Ustekinumab as a treatment for CD[147]. Thus, the upregulation of *Il17a* and *Il22* expression under CVS indicates antigenic stimulation of myeloid sources of IL-1β, IL-6, and IL-23, possibly a result of dysbiosis, leading to activation of Th17 and ILC3 populations for protection of the mucosal barrier. As per histological analysis (Fig. 5), the ileal and colonic barriers appear to be intact and unchanged at the end of CVS, making it unlikely that overt epithelial injury is the possible trigger.

The transcriptional analysis of the ileum at the end of CVS has provided useful preliminary data and raises questions about the origins and consequences of the upregulated immune pathways. Though these data are consistent with the hypothesis that CVS promotes an inflammatory disease-state environment, there are limitations to consider. First, these transcriptional data are only representative of male mice. Female mice have not been evaluated for the expression of *Il1β*, *Il6*, *Il17a*, *Il22*, or *Il23*. If these transcriptional modulations are GC-dependent, it can be expected that female mice will not show the same phenotype as they do not show the same sensitivity to CVS as male mice (Fig. 6b). Pinpointing the mechanisms behind stress-induced immunomodulation is challenging and would require consideration of other stress mediators to isolate the effect of GCs. Namely, catecholamines such as NE and the neurotransmitter ACh have both been key effectors of stress-induced immuno-modulation in the gut[82,111]. Thus, I suggest consideration of multiple stress mediators and exploring whether the sex differences are restricted to GCs or if the dichotomy extends to other elements of the stress response. Moreover, to further probe the transcriptional changes which occur under CVS, and to isolate the sole changes caused by GCs, I propose the following RNA sequencing experiment on the terminal ileum: four groups, comprised of one uncontrived control group, one CVS group, one

CVS group administered mifepristone (RU486) which is a GR antagonist, and one control group also administered mifepristone. This would serve to both outline the global effects of CVS, as well as provide a pre-emptive filtering of GC-related changes.

After examining the sole effects of CVS in otherwise uncontrived mice, I sought to design a one-two hit model wherein I would assess the effects of CVS on the severity of an additional insult to the intestinal barrier, with a view to design a microbiome-centered CD model of chronic stress. Guided by our previous findings, I chose to pair CVS with our established AIEC colonization mouse model[19]. It should be noted that this was done in female mice. Similar to uninfected experiments, stressed AIEC infected mice did not show weight loss or stagnation (Fig. 14). In contrast to our previous findings which showed a profound expansion of AIEC under acute stress, AIEC in the current study did not differentially persist in CVS-exposed mice (Fig. 15). Additionally, while the $CD90^{+}ROR\gamma t^{+}$ compartment is uniquely susceptible to GC-based apoptosis under acute stress and AIEC expansion[19], these populations were unchanged in AIEC-infected CVS mice, at least on day 21 during the CVS protocol (Fig. 16). While this does not indicate any specific immunomodulatory effect of CVS, it does suggest that CVS is divergent in its effects from acute stress. Curiously, while CVS in naïve mice appeared to cause a significant increase in LCN2 (Fig. 10), the stool content of LCN2 in AIEC-infected stressed mice did not show a significant increase (Fig. 17). This may be due to the AIEC colonization eliciting a mild increase in LCN2 in both control and stress groups which masks the subtle, but significant, increase observed in naïve mice.

Lastly, the CVS model has technical limitations that may challenge the interpretations drawn. One of these limitations is the possibility for contamination of the mouse microbiome as a result of frequent handling and the use of multiple external vessels for stress for 21 days, as demonstrated in the 16s rRNA sequencing experiments (Fig. 7). Another drawback is the

impractical nature of the model, which entails commitment to rigid timepoints multiple times a day for 21 days, leaving a large margin for variability across experiments as a result of necessary changes in scheduling of stressors. This is further complicated in experiments necessitating regular administration of chemical antagonists such as mifepristone. Thus, I have been evaluating an alternative model using repeated restraint stress (RRS), which has been used in other studies on the effects of stress on the intestinal barrier (Fig. 3)[109,111]. In this model, I subjected AIEC-colonized mice to 3 hours of restraint stress per day over the course of 7 days. Over the course of repeated restraint stress, the stressed group showed a significant weight loss in comparison to the control group (Fig. 18). While significant weight loss suggests defects in the intestinal barrier, the stressed mice did not show a differential persistence of AIEC in comparison to the control group (Fig. 20). Thus, CVS and RRS indicate that chronic stress does not alter the host's liability to increased colonization by AIEC, but this does not exclude the potential for chronic stress to affect the pathogenic behaviour of AIEC in the gut. Histological analysis of control and RRS-exposed mice showed that both groups experienced mild epithelial erosion and leukocyte infiltration, likely a result of the AIEC colonization. However, the RRS group was showing a larger increase in leukocyte presence (Fig. 19a), and generally trended toward a more global spread of barrier defects as opposed to the focal damage seen in the controls. RRS has the potential to be a more practical and controlled model for the study of the effects of chronic stress on the microbiome, and the model can be further tailored toward scrutinizing chronic stress in the context of CD by applying RRS to IL10KO mice.

CONCLUSION

Chronic stress has been long observed to maladaptively influence IBD activity, and is a significant predictor of CD relapse. The biological basis of the link between stress and inflammation has been attributed to GCs and catecholamines, which exert a myriad of maladaptive immunomodulatory changes on mucosal immunity in the gut. However, the role of the microbiota within the gut-brain axis under stress is only just emerging as a central focus in the field. In this work, I aimed to unravel the connections between chronic stress, changes in the ileal barrier, and changes in the microbiota, with a view to connect these changes to form a microbiome-immune-brain axis.

It is important to note that a formal distinction between acute and chronic stress does not exist for humans nor for preclinical models. Hence, acute and chronic stress are not discrete categories; the effects of stress may exist on a spectrum that gradually changes with the progression of time, or there could be a key physiological "switching" moment in which acute stress passes a threshold into chronic stress. There is a gap in our understanding of the differences in pathology between acute and chronic stress which should be considered when interpreting results from preclinical models. The impact of CVS on the intestinal microbiota is not yet clear, but this work contributes to the basic science which shapes the rationales behind CD management regimens and treatments.

MATERIALS AND METHODS

All research was reviewed and approved by the Animal Review Ethics Board (AUP 20-12-41) at McMaster University and conducted in accordance with standards of the Canadian Council of Animal Care.

Animals and infections

Six- to eight-week-old C57BL/6N male and female mice were purchased from Charles River Laboratories and housed in Level 2 biohazard containment under specific pathogen-free barrier conditions and maintained on a 12 h light: 12 h dark cycle. One day prior to colonization with AIEC NRG857c, mice were treated with 20 mg of streptomycin by oral gavage. Mice were orally infected with 2×10^9 CFU of AIEC NRG857c in 0.1 mL sterile PBS. Mice were sacrificed by cervical dislocation.

Bacterial Cultures

AIEC NRG857c was grown overnight shaking in lysogeny broth (LB) supplemented with chloramphenicol (34 μg/mL) and ampicillin (200 μg/mL). Culture was prepared by centrifugation for 10 min at 4,000 × g followed by resuspending the pellet in sterile phosphate buffered saline (PBS) to a concentration of 2×10^{10} colony forming units (CFU)/mL.

Bacterial enumeration

Fecal samples were collected weekly during chronic variable stress starting at Day 1 in Mixer Mill safeseal tubes containing 1 mL sterile PBS. Samples were homogenized using a Mixer Mill for 5 min at 30 Hz. Homogenates were either plated neat or serially diluted at 10×, 10^2×, 10^3×, 10^4×, 10^5×, and 10^6× and plated on LB agar supplemented with chloramphenicol (34 μg/mL) and ampicillin (200 μg/mL). Plates were incubated overnight at 37° C.

Chronic variable stress

Stressed mice (*n*=4) were subjected to one light cycle stressor between 9:00h-16:00h and an overnight stressor 17:00h-7:00h for 21-24 days. Control mice (*n*=4) remained uncontrived for the duration of the protocol (Fig. 2). AIEC-infected mice began the protocol at day 5 post-infection. Light cycle stressors include 15 min forced swim in 30°C water; 1h cage shake at 80 rpm; 3h social isolation; and 1h restraint stress. Dark cycle stressors include disrupted light cycle; 45° cage tilt; damp bedding; food deprivation; and water deprivation. At week 3 (D22) mice were sacrificed by cervical dislocation and intestinal tissue was harvested for flow cytometry, transcriptional analysis, or bacterial enumeration.

Repeated restraint stress

Stressed mice (*n*=4) were placed in well-ventilated restraint tubes for 3h a day between 9:00h-16:00h in a separate procedure room. Control mice (*n*=4) were left uncontrived in their home housing room (Fig. 3).

Immunohistochemistry

At the time of sacrifice, ileum, cecum, and colon tissue were placed in ice cold 1× PBS. Ileum and colon were cut longitudinally, washed in 1× PBS and rolled with the mucosa facing outwards, and the cecal tip was cut from the cecum. Tissue sections were placed in cassettes and fixed for 72h in 10% formalin at room temperature, then stored in 70% EtOH at room temperature until paraffin embedding. Sections of 5 μm thickness were sliced for staining with hematoxylin and eosin (H&E) at McMaster University Histology Services. Slides were imaged using a Zeiss Axio Imager with 10× and 40× objective. Tissue sections were scored by a blinded third party.

ELISA protein quantitation

Stool was collected once a week in pre-weighed Mixer mill safeseal tubes, re-weighed post-collection, and suspended in 0.5 or 1 mL sterile PBS. Samples were homogenized using a

Mixer Mill for 5 min at 30 Hz. Fecal Lipocalin-2 was quantified using a mouse lipocalin-2/NGAL Duoset ELISA kit (R&D systems; DY1857) as per manufacturer's instructions.

Plasma corticosterone quantitation by LC-MS

Plasma samples were frozen at –80°C until processing. Corticosterone was extracted by protein precipitation. A corticosterone standard and corresponding deuterated internal standard (d8- corticosterone) were used to quantify the corticosterone in the experimental plasma samples. The internal standard was added to all calibration standards, test samples, and validation samples. To prepare the calibration curve, the ratio of the average area of the analyte response to the average area of the internal standard response for each of the calibration standards injected was plotted against the ratio of the calibration standard concentration to the IS concentration. The internal standard was used to account for instrument variability, matrix effects, and sample recovery.

Bulk tissue RNA extraction

At the time of sacrifice, tissue samples were taken from the distal ileum (~0.5-1 cm) and placed in Mixer Mill safeseal tubes containing 900 uL TRIzol (Invitrogen) on ice for RNA extraction. Samples were homogenized with beads using a Mixer Mill for 5 min at 30 Hz. For each sample, 400 uL chloroform was added and mixed by inverting, followed by incubation on ice for 2 min. Samples were then centrifuged for 5 min at 4° C and 12,000 rcf. The upper aqueous phase was removed by micropipette and added to tubes containing 500 uL isopropanol and mixed by inverting, then incubated at -20° C for 10 min followed by centrifugation for 5 min at 4° C and 12,000 rcf. The supernatant was then removed, and the pellets were washed by adding 950 uL 70% EtOH with centrifugation for 5 min at 4° C and 12,000 rcf. The supernatant was removed and pellets were dried in a biosafety cabinet for ~5 min. The pellets were resuspended in 100 uL 100% isopropanol and 20 uL 5M NaCl. Samples were incubated at -20° C for 10 min followed by

centrifugation for 5 min at 4° C and 12,000 rcf. The supernatant was removed and 200 uL of 70% EtOH was added to wash pellets followed by centrifugation for 5 min at 4° C and 12,000 rcf. Supernatant was then removed and RNA pellets were allowed to air dry for ~5 min. RNA pellets were resuspended in 100 uL nuclease-free water. RNA concentration and purity was assessed with a NanoDrop spectrophotometer.

16s ribosomal RNA sequencing

Ileal contents were collected from control and stressed mice in cohorts sacrificed on days 0, 14, and 21. The contents were placed in sterile tubes containing NaPO4 and guanidine thiocyanate/EDTA with sterile homogenization beads. Samples were sent for processing and sequencing at the Farncombe Metagenomics Facility at McMaster University. 16s rRNA v3v4 regions were Illumina sequenced. 16S data analysis was undertaken in R v4.4.1[157]. The phyloseq[158] and tidyverse[158] packages were used for data organization, and ggplot2[160] for visualization. 16S rRNA amplicon sequence variant (ASV) tables were cleaned as follows: ASVs were assumed to be host sequences and removed if they were not assigned to Kingdom Bacteria or Kingdom Archaea, if they lacked a Phylum assignment, or if they were assigned to Order Chloroplast or Family Mitochondria. ASVs were clustered to 99% identity using DECIPHER[161], taxonomy was re-assigned in the same manner, and host sequences were once more removed. Any OTUs with mean abundance ≤10 reads were removed. No samples had fewer than 5,000 reads, so all samples were retained for analysis. When calculating alpha diversity, all samples were rarified to the lowest sample read depth before calculating Shannon and Inverse Simpson diversity metrics. Bray-Curtis dissimilarity was calculated on unrarefied data normalized by read depth. Aitchison distances are the Euclidean distances between samples that have been centre-log ratio transformed. Permanova tests were conducted using the adonis2 function in the vegan package[162].

Reverse transcription-quantitative polymerase chain reaction

Reverse transcription was performed to convert RNA extracted from the distal ileum to complementary DNA (cDNA) using a cDNA SuperMix (Quanta Biosciences). Roche Lightcycler 480 II was used to perform qPCR using SYBR green SuperMix (Quanta Biosciences). All primers used are listed in Table 1. All samples were normalized to the housekeeping gene *Rplp0*. Fold gene expression in stressed mice relative to control mice was evaluated using the $2^{-\Delta\Delta CT}$ method, then a Log base 2 transformation was applied.

Lamina propria mononuclear cell (LPMC) isolation and flow cytometry

At the time of sacrifice, intestinal tissue was harvested and placed in cold RPMI 1640 supplemented with 10% v/v heat-inactivated FBS. Tissues were cut longitudinally, minced into ~5 mm pieces, and placed into 20 mL Buffer A (HBSS -/-; 50mM 7.5 pH HEPES; 1mM DTT) and agitated in a hybridization oven at 37°C for 15 min (30 rpm). The tissue was strained in a 100 µm strainer and placed into 20 mL Buffer B (HBSS -/-; 10% v/v heat-inactivated FBS; 10mM EDTA) and agitated for 15 min in hybridization oven at 37°C (20 rpm). Tissue was strained in a 100 µm strainer and placed into 5 mL digestion buffer (RPMI 1640; 5% v/v heat-inactivated FBS; 0.5mg/mL DNase I; 40 µg/mL Liberase) then incubated in a hybridization oven at 37°C for 35 min with agitation (30 rpm). Tissue was strained in a 70 µm cell strainer and supernatant was retained. Remaining tissue pieces were crushed through the strainer while washing strainer with RPMI 1640 up to a volume of 45mL. Cell suspension was centrifuged at 1300×g for 10 min at 4°C. Supernatant was decanted and pellet was resuspended in 6 mL 40% Percoll and overlayed on top of 3 mL 70% Percoll. The Percoll gradient was centrifuged at 2500 × g with acceleration and brakes at 0 for 30 min. Top debris layer was aspirated using a serological pipette, then all 40% Percoll (top layer) was aspirated to retrieve LPMCs. Cell suspension was expelled into 30 mL RPMI 1640 with 10% v/v heat-inactivated FBS and centrifuged at 1300 × g for 10 min at 4°C.

Supernatant was decanted and pellet was resuspended in cold 1× PBS. The single-cell suspensions were distributed into V-bottom 96 well-plates and centrifuged at 1300 × g then washed with 1× PBS. Cell pellets were resuspended in 25 µL of 10 µg/mL CD16/32 mAb and incubated in the dark at 4°C for 10-15 min, then 25 µL of surface-staining antibody cocktail was directly added to the cells and incubated in the dark at 4°C for an additional 20 min. All antibodies were diluted at 1:200 unless stated otherwise. Live/Dead Fixable Near-IR stain (1:1000) (Invitrogen); CD45 (BV421); CD3 (AlexaFluor700); CD4 (PE); CD8 (PE-Cy5); CD25 (APC); lineage exclusion (Ter119, Gr1, CD3, CD11b, B220) (FITC) (10 µL per test); CD90 (PE-Cy7). Following surface staining, cells were washed twice with 1× PBS then fixed and permeabilized with the Foxp3 transcription factor staining kit (eBioscience) for 20 min at 4°C in the dark. Following permeabilization, cells were washed twice with 1× wash/permeabilization buffer (eBioscience) and intracellularly stained with anti-RORγt (APC) (1:20) or Foxp3 (PE-Cy7) (1:50) diluted in 1× wash/permeabilization buffer for 1h in the dark at 4°C. Cells were then washed twice with 1× wash/permeabilization buffer and resuspended in 1× PBS. Data were acquired using a BC Cytoflex LX cytometer (Beckman Coulter) with CytExpert software and analysis was carried out with FlowJo Software (v10.9) (BD Life Sciences).

Statistical Analysis

Data were recorded with Microsoft Excel (v16) and analyzed using Prism GraphPad (v10) for all experiments.

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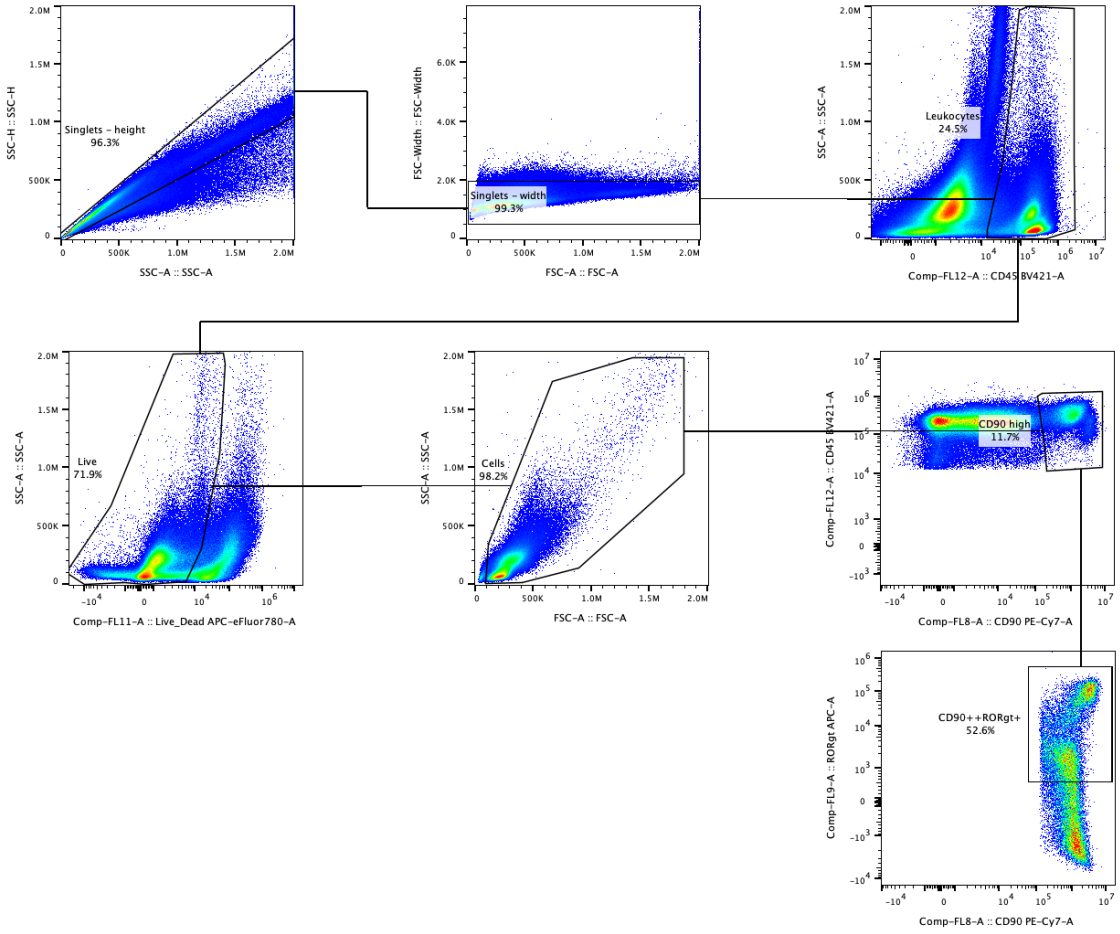
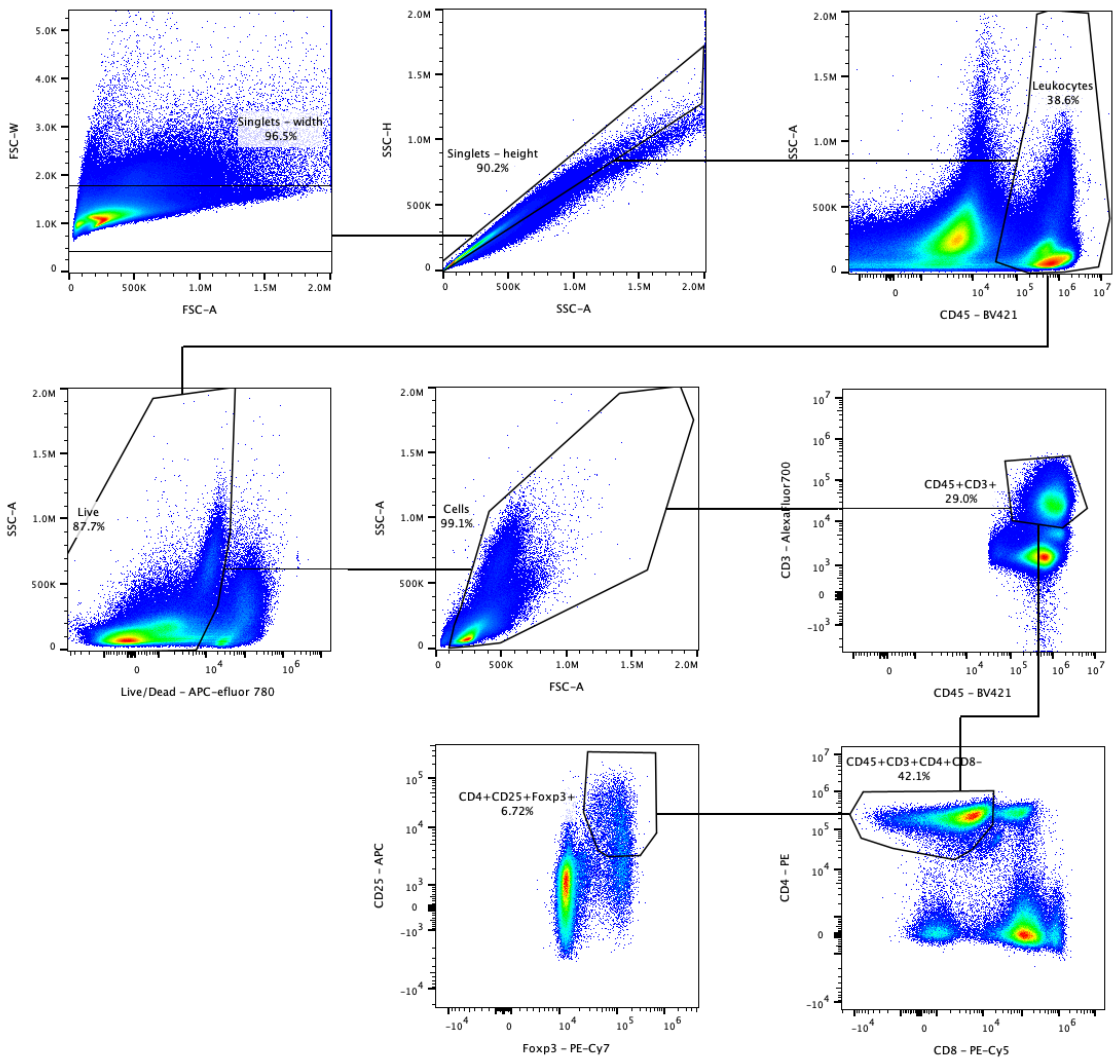
APPENDIX

Gene	Forward primer sequence (5'-3')
Rplp0	CCAATAAGGTGCCAGCTG
Il1β	AAGATGAAGGGCTGCT
Il6	AAGAGTTGTGCAATGGC
Il17a	ACTCTCCACCGCAAT
Il22	GGAGTCAGTGTAAGGA
Il23	GCAAGCAGAACTGGC

Table 1 – RT-qPCR primer sequences.



Supplementary Figure 1 – CD90^{+} flow cytometry gating strategy.



Supplementary Figure 2 – T_{reg} flow cytometry gating strategy.