PSYCHOLOGICAL STRESS IMPAIRS HOST IMMUNE DEFENCE AGAINST AIEC

PSYCHOLOGICAL STRESS DRIVES AN ABERRANT IL-22 AND NUTRITIONAL IMMUNE RESPONSE, FAVOURING CROHN'S DISEASE-ASSOCIATED PATHOBIONTS

Alexandra A. Parco, B.Sc.

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TITLE: Psychological Stress Drives an Aberrant IL-22 and Nutritional Immune Response, Favouring an Expansion of Crohn's Disease-Associated Pathobionts

AUTHOR: Alexandra Parco, B.Sc. (McMaster University)

SUPERVISOR: Dr. B.K. Coombes

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Lay Abstract

Crohn's disease (CD) is an inflammatory disease of the gastrointestinal tract resulting from an exaggerated immune response. CD patients often report a relapse of symptoms following a period of psychological stress and are at an increased likelihood of having pro-inflammatory strains of *E. coli* within their gut. Here, we use a model of restraint stress to investigate how psychological stress modulates the abundance of bacterial species associated with CD. We found stress results in the limitation of essential nutrients, allowing for an outgrowth of *E. coli*. Further, stress hormones lead to the loss of a protective immune response in which *E. coli* expansion can be prevented by blocking these hormones or restoring immune signalling. Together, we conclude that stress leads to immune cell death and creates an iron limited environment that favours *E. coli* expansion. Such work begins to uncover the functional consequence of stress and its' role in disease progression.

Abstract

Crohn's disease (CD) is an inflammatory disease of the gastrointestinal tract attributed to an aberrant immune response to environmental and microbial triggers. Individuals with CD exhibit an enrichment of pro-inflammatory strains of Adherent-Invasive *E. coli* (AIEC) and often report a relapse of symptoms following a period of acute psychological stress. Despite a known immunosuppressive role, the mechanism by which stress contributes toward the development and progression of intestinal inflammation remains unknown. Here, we use a well characterized model of restraint stress to investigate the influence of psychological stress on host protection against a CD-associated strain of AIEC. We found that stress results in profound intestinal dysbiosis, allowing for a complete dominance of Enterobacteriaceae. Interestingly, while stress alone drives a state of low-grade inflammation and loss of barrier integrity in the gut, in the presence of a pathobiont strain of AIEC, stress drives a substantially heightened inflammatory response which exacerbated the resultant loss of barrier integrity. Moreover, we have found stress induces an augmented nutritional immune response, providing AIEC a competitive niche against commensal bacteria lacking alternative methods of iron uptake. Further, we see that stress-induced glucocorticoids mediate broad apoptosis of the $CD45^{+}CD90^{+}$ lymphocytic population in the gut. The loss of this population prevents an appropriate IL-22 mediated response to dysbiosis. Accordingly, blocking glucocorticoid signalling or exogenous administration of IL-22 prevents the stress-induced expansion of AIEC. This work underscores the complex nature of psychological stress such that the combination of iron limitation and glucocorticoid mediated immune attrition are simultaneously required

for the stress-induced expansion of AIEC. These findings present novel insight into the mechanistic consequences of glucocorticoid signalling on impaired immune function and the provision of an inflammatory environment, resulting in a distinct impact on CD susceptibility. As such, deeper insight regarding the complex underpinnings of CD will assist in efforts to design representative models and will strengthen the discovery of targeted therapeutics.

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List of Abbreviations and Symbols

AIEC – Adherent Invasive *Escherichia coli*

AMP – Antimicrobial Peptide

CD – Crohn's disease

CRF – Corticotrophin Releasing Factor

DSS – Dextran Sodium Sulphate

Fut2 – Fucosyltransferase 2

HMOX-1 – Heme Oxygenase 1

HPA – Hypothalamic-Pituitary-Adrenal

HPX - Hemopexin

IL – Interleukin

ILC – Innate Lymphoid Cell

Lcn2 – Lipocalin

Muc2 – Mucin2

STAT-3 – Signal Transducer Activator of Transcription – 3

TLR – Toll Like Receptor

Declaration of Academic Achievement

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Conceptualization: C.R.S. & B.K.C.

Methodology: C.R.S., **A.A.P.**, and B.K.C.

Investigation, Validation, Formal Analysis: C.R.S. & **A.A.P.**

Visualization and Writing - Original Draft: C.R.S. & **A.A.P.**

Investigation: J.D., W.E., J.J., and E.F.V.

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Introduction

Crohn's disease etiology

Crohn's disease (CD) is an inflammatory disease of the gastrointestinal tract and is attributed to a hyperactive immune response towards a variety of environmental and microbial triggers[1-3]. The incidence of CD continues to rise globally, disproportionately affecting developed nations, such as Canada. However, there is growing evidence of emergent disease in the developing world, with the prevalence of cases growing in Asia, South America, and Africa[4]. Currently, over 270 000 Canadians are living with CD and consequentially, the economic burden of CD in Canada continues to rise, amounting to over 1.2 billion dollars per year[5]. CD follows a relapse and remitting disease course in which patients experience long periods of disease quiescence followed by a relapse of intense gastrointestinal inflammation. During the course of a disease flare, intestinal inflammation will symptomatically manifest as severe abdominal pain, diarrhea, cramping, weight loss, and patient malnutrition. Although the symptoms of CD can be managed by anti-inflammatory or immunosuppressive therapies, many frontline treatments remain expensive and have a high rate of non-responsiveness[6]. Further, many patients will eventually require surgical intervention in order to address advanced complications such as gastric fistulas and abscesses[7]. As such, deeper insight regarding both the drivers of CD and their combinatorial effects on the development and progression of intestinal inflammation will be important in uncovering new avenues of treatment.

As an inflammatory disease, a unique combination of genetic susceptibility, lifestyle factors, and microbial interactions play a contributing role in disease progression and the manifestation of pathological changes within the gut. Specifically, individuals with CD exhibit a reduction in microbial diversity[8-10] and a concomitant increase in the abundance of pathobiont strains of bacteria thought to drive inflammation[11,12]. Notably, patients exhibit an enrichment of strains of Adherent-Invasive *Escherichia coli* (AIEC), although the specific contribution of AIEC to intestinal inflammation and disease progression remains elusive and represents an essential area of research. Additionally, patients will often report a relapse of gastrointestinal symptoms following a period of psychological stress[13,14]. Accordingly, gaining a comprehensive understanding of the individual and combined influence of microbial constituents and exposure to psychological stress on intestinal immune function will offer an increasingly nuanced view of the progression of CD, potentially uncovering novel avenues for treatment.

AIEC in relation to CD

The manifestation of CD-related inflammation is often associated with alterations in the microbial constituents of the intestinal microbiome, resulting in an overall reduction in species diversity and substantial bacterial dysbiosis within the inflamed regions of the gastrointestinal tract[8-10]. Contributing to a loss of species diversity within the intestine, CD is often associated with a reduction in the levels of Clostridiales, coupled with a corresponding increase in the abundance of intestinal Proteobacteria species[8,10]. Specifically, CD patients exhibit a disproportional enrichment for strains of AIEC,

particularly within the ileal mucosa. As a pathobiont in nature, AIEC can be found asymptomatically within healthy individuals, however, constitutes only a minor constituent of the microbiome of non-CD patients. Conversely, AIEC is six-times more likely to be isolated from an intestinal tissue sample collected from a CD patient and often represents a dominant member of the inflamed intestinal environment[15,16]. As such, despite being a largely innocuous resident of the microbiome under homeostatic conditions, AIEC is capable of becoming overtly pathogenic when coupled with the various inflammatory changes associated with CD[17,18].

By definition, AIEC strains are able to adhere to and invade epithelial cells through the use of actin microfilaments and microtubules[16]. Additionally, AIEC strains acquired the ability to survive and replicate intracellularly within the resident macrophages of the intestine[16,19]. Due to the intimate proximity of AIEC to epithelial cells, this prolonged interaction allows for the continual engagement of host immune sensors within the gut, as such AIEC strains have been found to be more pro-inflammatory when compared to commensal strains of *E. coli*[20-23]. Further, it has been demonstrated that the presence of AIEC augments intestinal damage when coupled with a secondary insult such as dextran sodium sulphate (DSS), an induced model of colitis. Specifically, AIEC greatly increases the secretion of pro-inflammatory cytokines induced following DSS, resulting in increased histopathological changes[24,25]. Accordingly, AIEC thrives under states of intestinal inflammation and takes advantage of the competitive niche derived from a hyperinflammatory immune response. As such, studies have demonstrated an enrichment

of AIEC in a variety of inflammatory models designed to investigate the underlying risk factors commonly associated with CD predisposition, including antibiotic use[26], secondary enteric infections[23], western diet[27], and genetic models of disease susceptibilty[28]. Accordingly, the ability of AIEC to augment the host inflammatory response when challenged with various environmental insults is thought to be a contributor to disease severity in CD patients, whereby the resulting pro-inflammatory environment propagates a loss of bacterial diversity and exacerbates dysbiosis within the gut microbiome[17,25]. As such, it is thought that the colonization of AIEC may promote further microbial dysbiosis and potentiate symptoms of CD.

Immunological consequence of psychological stress

Moreover, in addition to shifts in bacterial communities within the gut microbiome, lifestyle factors such as psychological stress can play a decisive role in the manifestation and progression of CD. Accordingly, patients will often report a relapse of CD-associated symptoms following a period of heightened psychological stress[13,14,29,30]. Following an initiating stress event, the body quickly mounts a controlled hormonal response in order to mobilize the required energy to respond to the stressor and eventually return the body to a state of homeostasis. Namely, the stress response largely depends on the deployment of the hypothalamic-pituitary-adrenal (HPA) axis and the norepinephrine driven response of the sympathetic nervous system **(Fig. 1a and b**). At the apex of the HPA axis, corticotrophin releasing factor (CRF) is produced by the hypothalamus, rapidly signalling for the release of glucocorticoids from the adrenal cortex[31]. Following release,

the hydrophobic glucocorticoid hormone will bind to the intracellular glucocorticoid receptor, promoting the translocation of the hormone-receptor complex to the nucleus[32] (**Fig. 1b**). As implicated by name, glucocorticoids function to mobilize stored energy in the form of glucose in order to mount a successful stress response. However, the glucocorticoid receptor is ubiquitously expressed and thus can have diverging downstream transcriptional effects depending on the target cell type[32]. Accordingly, the immunological consequence of stress is highly context dependent. As such, acute stress events can lead to a robust recruitment of the innate branch of the immune system, increasing Interleukin (IL)-6 production[33,34] and phagocytosis of invading microbes[35,36]. Opposingly, long term stressors are detrimental in the ability of the immune system to launch a successful response. As such, psychological stress is commonly considered an immunosuppressive event, resulting in heightened production of the immunotolerant cytokine IL-10, and promoting widespread apoptosis of the adaptive immune population[37]. Accordingly, as both innate and adaptive immune cells express receptors for stress signalling hormones such as CRF and glucocorticoids, chronic stress has the capacity to greatly modulate cellular trafficking, proliferation, cytokine expression, and overall immune function, often to the detriment of host immune protection[38-43]. Largely, the mechanisms underlying the physiological consequences of psychological stress on immune function, specifically glucocorticoid mediated immune attrition, is currently unknown and remains an unanswered question within the field.

Indeed, prolonged psychological stress often results in an altered and frequently suppressed immune response, whereby the ensuing immunotolerant environment prevents adequate pathogen control. Seemingly contradictory, chronic stress models have been demonstrated to propagate the microbial and inflammatory changes commonly associated with CD, including the reduction of species diversity within the microbiome[33]. Notably, models of restraint stress have demonstrated a marked increase in pro-inflammatory IL-6 production and a concomitant outgrowth of pro-inflammatory bacteria, such as *Enterococcus faecalis*[33,44]. Accordingly, the ensuing inflammatory environment and rearrangement of microbial communities have decisive consequences in colitis models. As such, there is an implicated role for stress in exacerbating disease severity in DSS-induced models of colitis[33]. Additionally, psychological stress has been demonstrated to negatively influence the ability of the immune system to maintain intestinal homeostasis. Interestingly, *ex vivo* stimulation of isolated innate lymphoid cells (ILCs) with dexamethasone, a synthetic glucocorticoid, impairs the ability of ILCs to produce the cytokine IL-22, a keystone defender against opportunistic pathogens[45]. As a result, psychological stress has the capacity to greatly alter both pro- and anti-inflammatory immune responses, and is therefore capable of drastically altering the composition of the intestinal microbiome[33]. Despite these known immunomodulatory effects, the immunosuppressant power of glucocorticoids has been leveraged to treat the general inflammation that is commonly associated with CD and currently remains a frontline treatment option. As such, a deeper insight regarding the role of psychological stress in

disease progression and contextual influence on intestinal immune function will be essential in efforts to design targeted therapeutics.

Intestinal innate immune defences

The immune system in the gut relies on both cellular and intrinsic immune barriers to defend against the outgrowth of opportunistic pathogens. In the gut, the host relies on a variety of innate defenses to prevent pathogenic bacterial colonization, including the epithelial barrier maintained by tight junctions, a thick mucus layer, and a robust array of antimicrobial peptides (AMPs) (**Fig. 2**), as well as a population of immune cells present within the lamina propria. Together, the mucosal immune system is constantly poised to prevent pathogenic bacterial attachment and colonization[46]. The gastrointestinal tract is covered by two layers of mucus secreted by the goblet cells within the gut. The outer layer supports a diverse community of commensal bacteria, whereas the denser inner layer facilitates the maintenance of spatial segregation between the microbiome and intestinal epithelial cells, thus preventing excessive immune activation and an ensuing inflammatory response[47,48]. The mucus covering the intestinal tract also contains an array of AMPs and digestive enzymes, including β-Defensins, Reg3γ, and Lipocalin (Lcn2). The presence of AMPs in the mucus similarly act to facilitate physical separation, limit bacterial colonization, and prevent exaggerated and deleterious immune responses[49]. Not surprisingly, defects in barrier function and spatial segregation are contributing factors in the progression of CD and deficiencies in Mucin-2 (Muc2), the major structural component of mucus, increases susceptibility to DSS-induced models of colitis[50]. As

such, at the height of CD disease activity, patients display a 70% reduction in the expression of Muc2 and an increased bacterial burden within the mucus layer[51-53]. In addition to maintaining spatial segregation between bacteria and the intestinal epithelium, epithelial cells utilize tight junction proteins to limit gut permeability and maintain the integrity of the epithelial barrier. As such, loss of tight junctions and the disruption of the innate barriers allows for bacterial dissemination, resulting in systemic inflammation. Through the activation of the mucosal immune system, bacterial leakage causes the recruitment of various immune cells and the production of pro-inflammatory cytokines, such as IFN-γ and TNF[54]. Potentiating this effect, TNF and IL-1β can additionally cause intestinal epithelial cells to alter the expression and localization of tight junctional proteins, promoting a further reduction in barrier integrity[55,56]. This resulting increase in intestinal permeability propagates bacterial translocation, creating a feedback loop that potentiates the facilitation of intestinal inflammation and resulting barrier damage.

Interestingly, psychological stress has been strongly implicated in a loss of barrier integrity, resulting in increased permeability across the epithelial barrier[57-59]. Signalling hormones released during stress, such as CRF, are capable of activating the degranulation of mast cells, leading to increased mucosal myeloperoxidase activity and pro-inflammatory TNF secretion along the length of the intestine[60,61]. The resulting release of immune proteases has been shown to disrupt tight junctions through the reduced expression of junctional proteins such as claudins and zonula occludins[60,62]. Through this disruption of junctional proteins and mucus production, stress has been reported to alter

gut permeability, allowing increased bacterial adhesion to epithelial cells, and facilitating increased diffusion of luminal antigens into systemic circulation[57,58,62,63]. As such, there is an emergent role for psychological stress in the breakdown of intestinal innate defenses and creation of a pro-inflammatory environment.

Dysregulated interactions between the mucosal immune system and luminal antigens plays a fundamental role in the development and progression of colitis. Previous studies have highlighted the importance of the microbiome in the pathogenesis of CD in humans[8-10]. Moreover, this is further demonstrated as antibiotic treatment ameliorates DSS-induced colitis in mice[18]. The innate immune system is able to recognize and respond to bacterial antigens through the engagement of Pattern Recognition Receptors, including Toll-Like Receptors (TLR). TLR4 is able to recognize lipopolysaccharides (LPS), the major component of the outer membrane of gram-negative bacteria, such as AIEC. Once activated by LPS, TLR4 is able to signal downstream to NF-κB in order to induce the expression of pro-inflammatory cytokines including TNF, IL-6, and IL-1β[64,65]. Given the pro-inflammatory nature of TLR signalling, TLR4 induction has been implicated in the pathogenesis of CD as a causative link between dysbiotic microbial populations and intestinal inflammation. Previous studies have shown that TLR4 receptors are upregulated in CD patients and have linked TLR4 signalling to microbial dysbiosis, favouring an outgrowth of pro-inflammatory bacteria[24,66]. Furthermore, TLR4 deficient mice display a reduction in macroscopic signs of colitis following DSS, inflammatory activity, and neutrophil recruitment[24,67]. Additionally, TLR4 signalling has

been heavily implicated in the immune response to stress. Several studies have demonstrated that stress hormones, including CRF, promote TLR4 expression, as well as the downstream phosphorylation of the transcription factor Signal Transducer Activator of Transcription (STAT)-3[37,59,68]. Indeed, TLR4 activation and resulting pro-inflammatory signalling in the context of stress contributes to further epithelial damage and increased gastrointestinal permeability[37,59]. Accordingly, understanding how psychological stress influences the innate immune system, is critical to understanding how stress induces a relapse of symptoms in CD patients.

The contextual role of IL-22 in bacterial infection and immunity

In addition to the intrinsic immune defences present at the intestinal epithelial barrier, the host relies on a diverse population of immune cells to maintain intestinal homeostasis and prevent the colonization of opportunistic pathogens. Over the last decade, the cytokine IL-22 has emerged as a central regulator of health and disease within the intestine and plays an essential role in coordinating an appropriate immune response to various types of intestinal dysbiosis[69-71]. Although IL-22 is constitutively produced at low levels, and is essential to maintaining intestinal homeostasis, the production of IL-22 is rapidly induced under states of infection in response to the TLR4 mediated induction of IL-23[69,72,73]. Produced by various members of the innate immune system, IL-23 correspondingly signals the downstream production of IL-22, predominantly by lamina propria-resident ILC3s[74,75] (**Fig. 3**). Additionally, IL-22 has been reported to be produced by various T cell subsets including T_H17 and T_H22 cells[76], as well as neutrophils[77], albeit to a lesser

extent. As a member of the IL-10 family of cytokines, IL-22 engages a two-subunit receptor with distinct tissue distribution[78]. While the IL-10Rβ receptor subunit is ubiquitously expressed, the IL-22R1 receptor is largely specific to nonhematopoietic cells[78], such as epithelial cells, allowing IL-22 signalling to impose broad transcriptional changes at mucosal sites. Accordingly, IL-22 drives the phosphorylation of STAT-3 in epithelial cells to induce the expression of a variety of antimicrobial products and promote tissue repair[70,75,79,80].

Central to its protective function, IL-22 promotes epithelial AMP production which functions to repel pathogenic bacteria, as well as safeguard microbial diversity within the gut. Most notably, murine studies have demonstrated that IL-22 signalling is crucial to mounting innate immune defense against *Citrobacter rodentium*, an attaching and effacing mouse pathogen[81-84]. Using a variety of model systems (genetic alblation[84], obesity driven loss of IL-23 signalling[81], loss of ILC3 development[83]), researchers have demonstrated the essentiality of IL-22 following *C. rodentium* infection. Indeed, IL-22 deficient mice exhibit increased intestinal damage, endotoxemia, and mortality[81,84], which can be mitigated through the delivery of exogenous IL-22[81] or direct delivery of the IL-22-inducible AMP, Reg3γ[84]. As such, AMP driven production presents a keystone role for IL-22 in immune response to bacterial infection (**Fig. 4**). Unsurprisingly, host pattern recognition receptors are capable of detecting various effector proteins secreted by the *C. rodentium* type II secretion system and play a defining role in the initiation of IL-22 secretion and epithelial repair following infection[85]. Pathogens such as *Salmonella*

Typhimurium have evolved mechanisms to exploit the distinct changes induced by IL-22 seen following infection. By restricting the growth of its commensal competitors and inducing a state of iron limitation, IL-22 driven AMP production can paradoxically provide *Salmonella* a distinct competitive advantage within the inflamed gut[86] (**Fig. 4**). As such, it is evident that contextual and controlled regulation of the IL-22 system is essential for maintaining homeostasis and preventing the development of an exploitable niche for pathogenic species of bacteria.

IL-22 driven immune defense and intestinal homeostasis

The ability to robustly induce the expression of AMPs is perhaps the most well characterized function of IL-22, and it is through enhanced AMP production that IL-22 maintains intestinal homeostasis (**Fig. 5a**). In response to microbial dysbiosis, IL-22 potently upregulates the production of AMPs by epithelial cells through engagement of the STAT-3 signalling pathway. STAT-3 activation induces the expression of key AMPs including, Reg3β[72,77,87-89], Reg3γ[72,88,89], Serum Amyloid[90], Lcn2[86,91] and Calprotectin[77]. As previously noted, the C-type lectin Reg3γ acts to maintain spatial separation between the constituents of the microbiome and the intestinal epithelium in order to limit excessive immune activation and inflammation[92]. Indeed, ablation of IL-22 signalling by means of antibody mediated depletion of ILCs and T cells (anti-CD90) or direct antibody neutralization of IL-22 results in leakage of commensal bacteria and systemic expression of the inflammatory cytokines, IL-6 and TNFα. This chronic low-grade inflammatory response, in the absence of IL-22, greatly increases host susceptibility to infection[87].

Furthermore, the essentiality of IL-22 is shown in response to the physical damage caused by DSS-induced colitis, whereby the resulting bacterial leakage from the gut lumen triggers a potent IL-22-dependent AMP response[72,88]. Indeed, mice lacking IL-22 are more susceptible to DSS-induced pathology and the exogenous delivery of IL-22 protects against epithelial damage[72]. Furthermore, delivery of the purified IL-22-dependent AMP, Reg3α, is capable of circumventing disease severity by limiting DSS-associated inflammation and associated epithelial damage[93]. Together, the selective pressures afforded by the IL-22-dependent induction of AMPs act to shape the constituents of the resident microbiome and function to maintain mucosal homeostasis.

In addition to the induction of a broad repertoire of AMPs, IL-22 functions to maintain homeostatic balance within the gut through the coordinated addition of sugar residues to the glycoproteins and lipids on the intestinal epithelium. The α(1,2) linkage of fucose groups by fucosyltransferase 2 (FUT2) provides an alternative fuel source to bacterial species and functions to maintain commensal populations, while preventing the establishment of an opportunistic niche for invading pathogens (**Fig. 5b)**[94]. Indeed, mice lacking IL-22 dependent sources of fucose are more susceptible to both *C. rodentium* and *Salmonella* infection[71,74,94]. Further, by favouring the growth of succinate-consuming species, IL-22-mediated N-glycosylation indirectly limits the infectious capacity of *C. difficile* through increased competition for essential metabolites[79]. As such, metabolomic changes mediated by IL-22 provide an additional fuel source for members of the

commensal population, allowing for maintained colonization resistance and homeostasis within the gut.

The IL-22 axis additionally functions to promote epithelial repair and maintain barrier integrity. As such, in the event of epithelial damage following various chemical and biological exposures (DSS[80,95], Muc2$^{-/-}$[96], high fat diet mediated inflammation[97]), there is a corresponding induction of the IL-22-STAT3 signalling pathway. The induction of the IL-22 pathway results in increased epithelial cell proliferation[95,96], mucus production[95], and is required to effectively initiate the DNA damage response[98] (**Fig. 5c)**. Opposingly, in the event of enteric infection, IL-22 can increase barrier permeability via an increase in claudin-2 expression[99,100], allowing for the expulsion of invading microbes[99]. As such, through diverging mechanisms, IL-22 acts as an essential defender of intestinal homeostasis both proactively by maintaining the commensal population and retroactively through the deployment of AMPs and barrier repair.

Interestingly, defects in the IL-22 signalling pathway are associated with the progression and severity of CD. Mutations in the IL-22 receptor subunit IL-10R1 are associated with early onset forms of CD[101] and IL-22 has been shown to be upregulated in both CD patients and DSS models of colitis in mice[102]. Additionally, *ex vivo* stimulation with stress hormones has been demonstrated to functionally impair the induction of the IL-22 axis, whereby glucocorticoids impede the ability of isolated ILCs to produce IL-22[45]. Accordingly, the role of IL-22 signalling in relation to both microbial dysbiosis, and

psychological stress remains an exciting area of research within the field and could greatly enhance our understanding of CD.

Nutritional Immunity

Amongst the most notable roles of IL-22 is its ability to induce the production of AMPs that act as metal scavengers in order to facilitate a state of nutritional immunity. Thus, through the sequestration and limitation of essential metals and ions, the induction of nutritional immunity potently limits bacterial replication and growth[103]. IL-22 robustly induces the expression of Lcn2, an AMP which functions to prevent bacterial acquisition of the iron bound form of the bacterial siderophore enterobactin[86] (**Fig. 5d**). Indeed, in the event of *Salmonella* infection, the host potently induces the production of Lcn2 in an IL-22 dependent manner[86,91]. Similarly, IL-22 can limit iron uptake and availability in the serum through the induction of hepcidin[104] and heme scavengers[105]. Indeed, in IL-22-deficient mice, following *C. rodentium* infection, the unregulated accumulation of free hemoglobin can contribute to increased pathogen growth by providing an iron source. Likewise, the restoration of nutritional immunity via IL-22 mediated induction or exogenous administration of the heme scavenger, hemopexin (HPX), can limit bacterial growth within IL-22-deficient mice[105]. Accordingly, IL-22 has been demonstrated to play a fundamental role in the regulation of nutritional immunity and the corresponding limitation of the colonization of opportunistic pathogens. In addition to IL-22 dependent mechanisms, the host rapidly circumvents the availability of essential nutrients in response to classical immunological cues associated with bacterial infection, including

pro-inflammatory signalling, TLR activation, and the unfolded protein response[103]. In an evolutionary arms race, pathogens have adapted to perform hemolysis, breaking down erythrocytes in order to release iron rich stores of hemoglobin. Accordingly, iron released via hemolysis is quickly sequestered from bacterial uptake through the engagement of nutritional immune defenses and the corresponding induction of haptoglobin and HPX. Additionally, the induction of hepcidin induces the internalization and degradation of the iron efflux transporter, ferroportin, on the surface of macrophages, preventing iron export following macrophage mediated erythrolysis[103]. Studies have identified a role for nutritional immunity in preserving the intestinal microbiota, maintaining the population of commensal species commonly associated with intestinal health, such as Bifidobacteria[106]. However, iron limitation can opposingly result in profound dysbiotic changes within the gut, resulting in a concomitant outgrowth of *Enterobacteriaceae* species that are capable of thriving under states of iron limitation[107]. As such there is mounting evidence that iron-associated microbial shifts can drastically influence intestinal homeostasis and have a resulting negative outcome on disease susceptibility. Indeed, hepcidin deficient mice exhibit increased susceptibility to models of DSS-colitis, an effect that was mitigated by oral administration of Bifidobacterium[106]. As such, there is a key role for the coordinated limitation of iron to prevent excessive pathogen growth in states of infection and ensure the maintenance of intestinal homeostasis.

Paradoxically, despite profoundly limiting bacterial availability to essential nutrients, the induction of nutritional immunity can inadvertently favour certain invading pathogens.

Specifically, *Salmonella* can evade nutritional host defenses through the use of alternative siderophores that are resistant to Lcn2 sequestration, thereby allowing them a distinct advantage during states of nutritional immunity[86] (**Fig. 5d**). Whereas commensal bacteria largely rely on the siderophore enterobactin to acquire iron, *Salmonella* and strains of AIEC possess the alternative siderophore salmochelin, a glycosylated form of enterobactin that is unable to be sequestered by Lcn2 under states of nutritional immunity. Indeed, *Salmonella* Typhimurium mutants that are incapable of producing salmochelin are drastically outcompeted by wildtype strains of *Salmonella* within wildtype mice but lose their colonization advantage in Lcn2 deficient mice[91], demonstrating a direct role for nutritional immunity in bacterial fitness. Indeed, probiotic strains of *E. coli* well equipt to acquire iron can thrive in the inflamed gut, impairing the competitive advantage conferred to *Salmonella*[108]. These findings highlight an essential role for iron limitation in the provision of an expansive niche for members of the *Enterobacteriaceae* family that encode a variety of nutrient acquisition mechanisms that can evade immune mediated states of nutrient limitation.

Hypothesis and Research Objectives

This work aims to investigate the combinatorial effects of psychological stress and the colonization of a CD-associated pathobiont strains of AIEC on intestinal dysbiosis. Evaluating these two risk factors associated with increased susceptibility to CD relapse, we will attempt to uncover novel interactions between the gut microbiome, the resident immune system, and psychological stress. Here, we leverage a characterized model of

psychological stress to investigate the resultant influence of stress and AIEC colonization on mucosal immune function.

Hypothesis: Psychological stress impairs host immune responses to a CD-associated strain of AIEC, providing AIEC a competitive advantage in the stress-exposed gut.

Objectives:

1. Identify the effect of psychological stress on microbial dysbiosis and bacterial burdens of a CD-associated strain of AIEC.
2. Investigate the impact of psychological stress on innate host defenses, specifically the ability of the intestinal barrier to maintain bacterial containment.
3. Investigate the role of iron limitation and the induction of nutritional immunity in the expansion of AIEC seen following stress.
4. Profile immune changes in response to psychological stress and determine the role of glucocorticoid signalling in microbial dysbiosis
5. Confirm the combinatorial nature of psychological stress and determine the contribution of IL-22 signalling and iron limitation to AIEC expansion

Summary of Key Findings:

Our results demonstrate that stress impairs the function of the innate intestinal barrier driving the induction of a mixed inflammatory response. Further, we demonstrate that stress induces an uncoupled mucosal immune response, where there is potent induction of

nutritional immunity, despite a concomitant loss of the IL-22 producing $CD90^+$ population of lymphocytes. We show that the loss of $CD90^+$ lymphocytes was mediated by the production of stress-derived glucocorticoids and could be reversed by blocking glucocorticoid signalling. Together, the resultant state of nutritional immunity coupled with impaired immune surveillance creates a permissive niche for uncontrolled AIEC expansion and increases susceptibility to DSS-induced colitis. Interestingly, the exogenous administration of IL-22 prevents the stress-induced expansion of AIEC and mitigates DSS-associated sickness. This work presents a deeper understanding of the functional consequence of psychological stress on intestinal immune function and presents an exciting role for IL-22 administration in correcting bacterial dysbiosis associated with colitis.

Results

Psychological stress promotes the expansion of ileal *Enterobacteriaceae*

Psychological stress is largely thought to be immunosuppressive in nature[37] and is thought to contribute to a worsening of disease symptoms in individuals with CD[14,109,110]. Further, previous reports have indicated that psychological stress can lead to modest alterations in the gut microbiota[33]. As bacterial dysbiosis is a contributing factor to intestinal inflammation in CD patients[8,15,111], we sought to profile microbial changes in the gut following a stress event. To do so, mice were restrained in well ventilated 50 mL conical tubes overnight, to elicit psychological stress[112,113], or had food or water removed as a relevant control (**Fig. 6a**). Following release from stress, we sampled the luminal contents from the ileum, cecum, and colon in order to investigate changes within the microbiome via 16S rRNA sequencing. Interestingly, there was a profound global expansion of *Enterobacteriaceae* throughout the intestine following psychological stress, despite being present in less than 1% abundance in uncontrived groups of mice (**Fig. 6b-d**). However, our 16S data suggests that *Enterobacteriaceae* expansion was most substantial within the ileum and dominated over 80% of the bacterial species present, suggesting that *Enterobacteriaceae* members are well equipt to gain a foothold in the stress microenvironment. Moreover, regardless of the intestinal compartment profiled the genus *Escherichia-Shigella* exhibited the highest proportional change in relative abundance following stress (**Fig. 6e-g**). Interestingly, many of the bacterial genera enriched following stress, namely *Enterococcus, Proteus,* and *Mucispirillum* (**Fig. 6e-g**), are similarly increased in patients with CD and in mouse models of spontaneous ileitis.

Accordingly, there is a corresponding significant reduction of diversity of the ileal microbiome (**Fig. 6h**) and general loss of diversity in the cecum and colon (**Fig. 6i-j**). Despite not reaching significance in the cecum and colon, all intestinal compartments clustered distinctly by PCA analysis when comparing the microbiomes of stress and control mice (**Fig. 6h-j**). Together these findings suggest that stress leads to distinct microbial changes and significant loss of species diversity, highly favouring an enrichment of *Enterobacteriaceae*.

Psychological stress impairs host control of CD-associated AIEC

Given the role of CD-associated pathobionts in exacerbating experimental models of colitis and driving inflammation[24,25], we were curious to see how psychological stress would influence the colonization of a strain of AIEC. To test this, mice were infected with NRG857c, an ileal isolate of AIEC, and bacterial burdens were allowed to stabilize for five days (**Fig. 7a**). Following AIEC colonization, mice were similarly restrained overnight or starved as a control. To determine if psychological stress similarly provides AIEC a competitive niche, AIEC burdens were investigated in the feces and following stress recovery. Six hours following release, there was a dramatic expansion of AIEC with in the feces, such that AIEC fecal burdens were 4-5 orders of magnitude higher than AIEC burdens measured in controls. However, the permissive niche granted to AIEC appears to be a transient effect, in which the burdens of AIEC are constrained to those of control following 24 h of recovery (**Fig. 7b**). Based on the finding that the host is able to normalize AIEC burdens following one stress event, we were interested to identify the

effect of repeated stress on bacterial expansion and retraction. To do so, AIEC colonized mice were exposed to overnight restraint stress weekly for a duration of one month. Indeed, there was a stereotyped outgrowth of AIEC in the feces following each stress event (**Fig. 7c**). However, whereas control mice were able to clear the fecal AIEC population to undetectable levels, there was a progressive disablement of the host response, such that stress exposed mice maintained high levels of AIEC within their feces throughout the course of the experiment (**Fig. 7c**). Together, these results suggest that acute stress creates a supportive environment for the uncontrolled expansion of AIEC and that repeated stress impairs the host's ability to restore intestinal homeostasis, ensuring a foothold for AIEC in the gut. To further these findings, we were interested to determine the geographic region in the intestine in which AIEC was present following stress. Immediately following stress, the cecum, colon, and four equal segments of the small intestine, with segment one being closest to the stomach, were collected for bacterial enumeration. Predictably, AIEC favourably colonized the lower portions of the digestive tract, namely the ileum, cecum, and colon, within control mice (**Fig. 7d**). However, there is a dramatic expansion of AIEC throughout the entire length of the gastrointestinal tract. Most interestingly, restraint stress allowed AIEC to flourish in the upper regions of the small intestine, including the jejunum and duodenum, that are not typically colonized by AIEC (**Fig. 7d)**. These findings suggest that overnight stress creates global environmental changes along the length of the intestine, creating a favourable niche for AIEC expansion. Based on the robust expansion of AIEC in response to psychological stress, we utilized 16S rRNA sequencing to identify the effect of stress on the AIEC colonized microbiome.

Indeed, stress led to a complete dominance of *Enterobacteriaceae* within the ileum (**Fig. 7e**), such that *Enterobacteriaceae* accounted for >90% of the bacterial sequences present. Accordingly, this robust expansion led to a marked reduction of community diversity within the ileum and distinct clustering of the microbial populations collected from control and stress mice (**Fig. 7f**). Together, these findings suggest that psychological stress leads to profound dysbiosis in the microbiome, specifically favouring the outgrowth of AIEC at the expense of microbial diversity.

Previous studies have highlighted that both stress and the presence of AIEC can increase the severity of models of colitis[17,25,33,114]. As such, we were interested to identify the influence of stress in a DSS model of colitis in the presence or absence of AIEC. To test this, mice were exposed to a low dose of DSS for five days, during which time they were exposed to one bout of overnight restraint stress, before being switched to water. Stress alone resulted in a modest weight loss that was quickly recovered within 24 h and mice rebounded to baseline weights before being introduced to water. Indeed, mice exposed to stress and DSS in the absence of AIEC lost ~10% of their body weight but were able to recover to their baseline weights (**Fig. 7g**). In contrast, DSS exposed stress mice that were colonized with AIEC exhibited profound weight loss, amounting to a loss of ~20% from their baseline weights. Additionally, these mice failed to recover to their initial weights even after 2 weeks of water exposure. These results highlight the combined ability of stress and pathobiont colonization in increasing susceptibility to colitis and driving DSS-induced sickness.

Exposure to acute psychological stress impairs ileal barrier function and exposes mice to invasive microbes.

Based on the ability of stress to induce the uncontrolled outgrowth of AIEC and resulting microbial dysbiosis, we were interested to identify transcriptional changes in the gut that could benefit AIEC expansion. Specifically, previous reports have highlighted the ability of AIEC to thrive in inflammatory environments and AIEC favourably colonizes the gut of CD patients within inflamed regions of the gastrointestinal tract[11,12]. Accordingly, we employed RT-qPCR to investigate changes in cytokine transcript expression following stress (**Fig. 8a**). In agreement with previous reports which define a role for stress in immune suppression[37], we see that our model results in a marked induction of IL-10 transcript expression (**Fig. 8a**). However, we simultaneously see an upregulation in transcript expression for a variety of pro-inflammatory cytokines including *IL-17*, *IFN-γ,* and *TNF*. As such, it is evident that overnight restraint stress creates a mixed inflammatory environment, robustly increasing the expression of suppressive cytokines classically associated with stress, in addition to enhancing the production of pro-inflammatory cytokines associated with T_H17 and T_H1 signalling.

Indeed, inflammatory signalling can detrimentally influence the ability of the host to maintain barrier function, resulting in increased gastrointestinal permeability[115,116]. To address this, we similarly investigated how stress modulated the expression of genes associated with barrier integrity using RT-qPCR. Exposure to psychological stress led to

a downregulation of the tight junctional proteins claudin and occludin, and reduced expression of mucins (**Fig. 8c**). In contrast, the expression of the gene *GATA-4*, which plays a role in barrier repair[117], was markedly induced following restraint. These finding suggest that stress impairs the maintenance of the intestinal barrier. To confirm the functional consequence of these findings, we investigated intestinal permeability using an Ussing Chamber system. Interestingly, regardless of the presence of AIEC, psychological stress significantly impairs barrier function and allows for increase paracellular uptake of radiolabelled Chromium (**Fig. 8d**). However, AIEC appears to further exacerbate intestinal permeability following stress when compared to naïve stress mice, highlighting the pathobiont nature of AIEC. Based on these results, it was predicted that impaired barrier function and increased gut permeability would result in bacterial dissemination from the intestinal tract to the liver, an organ which is typically devoid of bacteria under homeostatic conditions. Stress led to a significant increase in the levels of LPS, a membrane component of gram-negative bacteria, within the liver (**Fig. 8e**). Additionally, we identified an increase in systemic bacterial translocation to the liver. Whereas 60% of control mice had undetectable levels of bacteria present in their liver, only 25% of the livers of stress mice were devoid of bacteria (**Fig. 8f**), confirming a role for stress in enhancing intestinal permeability. To determine the immunological consequence of bacterial dissemination, we investigated the induction of the Pattern Recognition Receptor, TLR4. Indeed, following barrier breach in stress there is an exaggerated induction of *TLR4* transcript expression, resulting in an ~8-16-fold increase when compared to unstressed controls (**Fig. 8g**). Similarly, there is a robust increase in the

downstream transcript expression of *IL-6*, such that stress exposed mice exhibited a ~64-fold increase in *IL-6* expression (**Fig. 8h**). Together, these findings exemplify the ability of stress to disable essential innate barrier protection and drive systemic inflammation.

In response to bacterial leakage and engagement of TLR4, the host immune system quickly responds to minimize bacterial dissemination through the deployment of neutrophils to the site of infection. Accordingly, flow cytometric analysis reveals a robust recruitment of $CD45^+CD11b^+Gr1^+$ immune cells, a population consistent with neutrophils, to the ileum in response to stress (**Fig. 8i**). These findings were additionally confirmed using immunohistochemical staining for GR1, and indeed confirm an increase in the accumulation of $GR1^+$ cells to the ileum of stress exposed mice. Together, these findings suggest stress induces a profound impairment of intestinal barrier function and drives an ensuing inflammatory and innate immune response to bacterial dissemination.

Stress-induces nutritional immunity within the gut and provides a competitive advantage to AIEC

In events of bacterial dysbiosis and barrier breach, the host immune system robustly employs an arsenal of AMPs aimed to limit bacterial acquisition of the essential ions and nutrients required for replication[103]. Most notably, neutrophils recruited to the site of bacterial dissemination will produce Lcn2 to limit the uptake of iron bound siderophores, namely enterobactin. Based on the heighted abundance of $CD45^+CD11b^+Gr1^+$ neutrophil-like cells, we were interested to determine if the induction of nutritional immunity was

contributing to the outgrowth of AIEC following psychological stress. To do so, we first profiled the transcript expression of genes involved in iron uptake and metal restriction using RT-qPCR (**Fig. 9a**). Indeed, psychological stress resulted in an increased expression of Heme Oxygenase-1 (HMOX-1), HPX, and Haptoglobin, suggesting stress alters iron metabolism by reducing the availability of iron-bound heme within the intestinal lumen (**Fig. 9b**). Additionally, there is a robust induction of Lcn2 and the calprotectin subunits s100a8 and s100a9 transcript expression, two classical AMPs induced in states of nutritional immunity (**Fig. 9b**). Interestingly, we see that while Lcn2 concentration is highly increased throughout the intestinal tract (**Fig. 9c**), the protein levels of the s100a8 calprotectin subunit were most substantially elevated within the lower bowel (**Fig. 9d**). Given the dramatic induction of nutritional immunity, we were interested to understand the time course of the induction of iron limitation and bacterial outgrowth in the context of stress. Accordingly, we investigated key nutritional immune pathways at two timepoints during restraint (4 and 16 h) and two timepoints following release and recovery (6 and 12 h). It is evident that stress rapidly evokes a nutritional immune response such that there is a remarkable increase in *Lcn2* transcript expression within the ileum and liver as early as 4 h during stress (**Fig. 9e**). Further, *Lcn2* transcript expression peaks at the 4 h time point but remains elevated throughout stress and 6 h following stress release and recovery (**Fig. 9f**). Interestingly, the ileal burdens of AIEC followed a similar time course to those of Lcn2 expression, such that burdens are highly elevated immediately following stress and at 6 h of recovery but are quickly constrained to baseline by 12 h (**Fig. 9g**). Indeed, there is a moderate positive correlation between the

burdens of AIEC and the protein concentration of Lcn2 and S100a8. As a result, high bacterial burdens correlate with robust induction of nutritional immunity (**Fig. 9h**), creating distinct clustering between stress and control samples. Together, these results suggest a role for the robust activation of nutritional immunity and iron limitation following stress in the outgrowth of AIEC.

Based on the remarkable induction of iron limitation following stress, we were interested to determine the mechanism by which nutritional immunity could confer AIEC a competitive advantage following stress. Unlike members of the commensal population, AIEC is equipt with a variety of alternative siderophores to circumvent host mechanisms of iron limitation. Specifically, AIEC encodes the *iroBCDE iroN* gene cluster which encodes for the alternative siderophore salmochelin, a glycosylated form of enterobactin, that is unable to be bound by Lcn2. As such, we wondered whether the ability of AIEC to evade host defense and acquire iron through alternative mechanisms was providing a competitive advantage over enterobactin dependent commensal species within the inflamed gut. To test this, we competed a Δ*iroB* mutant deficient in its' ability to produce salmochelin against wildtype AIEC. Indeed, under control states with limited Lcn2 production, the mutant Δ*iroB* and wildtype competed equally (**Fig. 9i**). However, stress significantly altered this dynamic, such that the Δ*iroB* mutant was attenuated compared to wildtype AIEC (**Fig. 9i**), indicating that the stress environment selects for bacterial species that are adapted to acquire iron. Together, these findings show that the robust activation of nutritional immunity provides AIEC a distinct competitive advantage over

species that are unable to evade host defense through alternative iron acquisition mechanisms.

Psychological stress induces attrition of CD90$^+$ cells, resulting in defects in the IL-22 pathway

The profound induction of nutritional immunity and AIEC's inherent ability to thrive in inflammatory and iron deplete environments led us to question the contribution of iron limitation in the outgrowth of AIEC following stress. To study this, we exogenously delivered a nonlethal dose of the TLR4 ligand LPS to AIEC-colonized mice in order to induce a corresponding nutritional immune response. Indeed, LPS administration led to a significant increase in the concentration of Lcn2 within the ileum, increasing measured Lcn2 levels to those seen following stress (**Fig. 10a**). However, despite a robust induction of iron limitation, burdens of AIEC failed to expand to the same extent seen following psychological stress (**Fig. 10b**), indicating that the engagement of nutritional immunity alone is insufficient to mediate uncontrolled AIEC growth. Accordingly, we were interested to determine whether other immunological changes following stress were contributing to bacterial dysbiosis. Indeed, previous studies have identified an immunosuppressive role of stress, such that glucocorticoid signalling can induce lymphocytic apoptosis[37]. Accordingly, we identified a remarkable depletion of the intestinal CD90$^+$ lymphocytes following stress, comprising an aggressive attrition of both the T-cell and ILC immune population (**Fig. 10c**). These results suggest that

psychological stress was significantly attenuating the ability of the immune system to successfully respond to ileal dysbiosis due to cellular depletion.

To further investigate the role of glucocorticoid signalling in immune function following stress, we investigated the levels of corticosterone, the mouse glucocorticoid hormone, in the serum following stress. Expectedly, serum concentration of corticosterone is robustly induced at the midway timepoint (8 h) of our stress protocol, and the administration of the glucocorticoid receptor antagonist RU486 does not interfere with corticosterone production (**Fig. 10d**) Further, in agreement with the literature, the majority of $CD90^+$ cells express the glucocorticoid receptor and are thus susceptible to glucocorticoid mediated immune modulation (**Fig. 10e**). Having confirmed the ability of our model to induce glucocorticoid signalling, we were interested to determine the role of glucocorticoids on stress-induced immune depletion. Interestingly, when evaluated at the midway timepoint (8 h) the frequency of $CD90^+$ cells present in the ileum is similar between control and stress samples in both the presence and absence of the glucocorticoid receptor antagonist RU486 (**Fig. 10f**). However, we see that stress induces an increase in the proportion of $CD90^+$ lymphocytes that stain positive for Annexin V, an early marker of apoptosis (**Fig. 10g**). Further, the increase in early apoptosis seen in stress is preventable via RU486 treatment, suggesting a glucocorticoid dependent role for stress-induced apoptosis. In congruence with these findings, we identified a stress dependent reduction of $CD90^+$ lymphocytes both as a factor of total cells (**Fig. 10h**) and by absolute

number of $CD90^+$ lymphocytes (**Fig. 10i**), an effect that is similarly prevented by RU486 mediated glucocorticoid signalling blockade.

In response to the observed impairment in $CD90^+$ cell accumulation following stress, we were interested to investigate the consequential changes in cytokine signalling. Specifically, we investigated the IL-22 response to stress given the established role of IL-22 in restoring states of bacterial dysbiosis and induction of AMPs. In response to stress, there is a profound impairment in the IL-22 axis, such that the total number of $CD45^+CD90^+IL\text{-}22^+$ lymphocytes are robustly depleted following stress (**Fig. 10j**). Similarly, the defect in the IL-22 producing population appears to be glucocorticoid dependent as RU486 treated mice that are exposed to stress maintain the IL-22 producing immune population to levels comparable to controls. Indeed, the attrition of the IL-22 producing lymphocytes leads to a functional impairment in downstream IL-22 protein production. When stimulated *ex vivo* with r-IL-23, the upstream inducer of IL-22, stress exposed mice fail to induce a corresponding increase of IL-22 that is typified in control mice (**Fig. 10k**). However, blocking glucocorticoid engagement by delivering RU486 prior to stress restores the ability of r-IL-23 delivery to induce IL-22 production, despite exposure to overnight restraint stress. Together, these data identify a glucocorticoid mediated depletion of the $CD90^+$ population following stress and highlight a central defect in the IL-22 response to the resultant intestinal dysbiosis and outgrowth of AIEC.

Psychological stress leads to an unbiased loss of the CD90$^+$ immune population

Based on our findings that psychological stress contributes towards an extensive attrition of the CD90$^+$ population, we wanted to determine if this profound cell death was resulting in a phenotypic shift of the remaining CD90$^+$ population, and thus altering the immunological landscape within the small intestine. However, we found that despite the significant cell loss, the overall proportions of CD90$^+$TCRβ$^{+/-}$ cells remains highly similar between control and stress exposed mice (**Fig. 11a**). Further, we find that comparable proportions of CD4$^+$, CD8$^+$, and CD4$^+$CD8$^+$ T cells are present within the small intestine despite exposure to psychological stress (**Fig. 11b**). Having identified a defect in the ability of stress-exposed mice to produce a corresponding IL-22 response when stimulated with r-IL-23, we wanted to further determine if there was a disproportional loss in the IL-22 producing population. Similar to the overall T cell proportions, we note the proportion of the major producers of IL-22, namely T$_H$17 and ILC3s, are not influenced by exposure to stress (**Fig. 11c**). As such, we find that although stress results in a significant reduction of the CD90$^+$ immune population and IL-22 production present in the gut, this event does not bias the distribution of the remaining CD90$^+$ cells.

Combinatorial effects of nutritional immunity and immune depletion are responsible for intestinal dysbiosis

Having defined a role for stress and glucocorticoid signalling in the apoptosis of CD90$^+$ lymphocytes, we were interested to uncover the contribution of immune depletion to the outgrowth of AIEC. Interestingly, despite their keystone role in maintaining intestinal

homeostasis, depletion of the $CD90^+$ population or IL-22 neutralization failed to phenocopy the expansion of AIEC that is associated with exposure to restraint stress (**Fig. 12a**). Based on these findings and the apoptotic role of glucocorticoids, we were interested to understand the other underlying factors in stress that were driving microbial dysbiosis. To do so, we began by pre-treating stress exposed mice with the glucocorticoid receptor antagonist RU486. Interestingly, we see that in the absence of intact glucocorticoid signalling, AIEC fails to expand following overnight stress (**Fig. 12b**), highlighting an important role for glucocorticoid signalling in AIEC expansion. However, in the absence of $CD90^+$ cells or IL-22 signalling, RU486 fails to prevent AIEC outgrowth and bacterial burdens expand to levels similar to those seen following stress (**Fig. 12b**). These findings suggest that glucocorticoid mediated depletion of $CD90^+$ cells following stress impairs the host's ability to respond to the profound dysbiosis, preventing the appropriate induction of protective IL-22 mediate responses. Building upon the role of glucocorticoid immune depletion in AIEC outgrowth, we were interested to identify the contribution of nutritional immunity to bacterial dysbiosis. To investigate the combinatorial factors underlying stress, mice were treated with LPS in order to simulate a barrier breach event and engage nutritional immunity. As previously demonstrated, LPS alone is insufficient to drive the expansion of AIEC to stress exposed levels (**Fig. 12c**). However, when the $CD90^+$ population is depleted, LPS administration allows AIEC to expand to levels comparable to restraint stress (**Fig. 12c**). These findings highlight the combinatorial nature of stress, such that neither LPS nor CD90 depletion alone can drive dysbiosis. However, it is evident that both iron limitation and a dampened

host immune presence simultaneously provide AIEC a competitive niche, allowing for uncontrolled bacterial expansion.

IL-22 delivery corrects stress-induced intestinal dysbiosis

Given the glucocorticoid induced impairment of a protective IL-22 response to AIEC outgrowth, we were interested to determine if the reconstitution of IL-22 would prevent stress-induced dysbiosis. Accordingly, in order to test this notion mice were treated with IL-22-fc prior to overnight restraint stress and resulting tissue burdens were investigated. Interestingly, while AIEC expanded to expected levels in the isotype treated stress group, IL-22-fc treated mice exhibited significantly lower bacterial burdens of AIEC despite exposure to stress (**Fig. 13a**). These findings further underscore an important role of IL-22 in preventing AIEC from gaining a foothold in the stress exposed gut.

Based on these results, we were interested to uncover how IL-22 restoration was capable of preventing AIEC outgrowth. To begin, we investigated IL-22 signalling to downstream target cells by staining for phospho-STAT-3 on isolated ileal epithelial cells. Indeed, in agreement with a stress-induced decrease in IL-22 production, we found that IL-22 signalling to its' epithelial targets is significantly impaired following overnight restraint when compared to control mice (**Fig. 13b**). However, reconstitution of IL-22 prior to stress prevented a reduction in phospho-STAT-3 staining, increasing IL-22 signalling to levels comparable to controls (**Fig. 13b**). Having determined that IL-22-fc administration could restore downstream signalling to epithelial cells, we were interested to uncover the

transcriptomic changes that could contribute towards the prevention of AIEC expansion. To do so, bulk ileal tissue samples were investigated by RNA-sequencing. Our findings indicate that stress leads to distinct changes in the intestinal landscape and that IL-22-fc treatment can normalize many of these transcriptional changes (**Fig. 13c**) Specifically, the pro-inflammatory environment defined by stress is ameliorated by IL-22-fc treatment and there is a robust increase in the expression of key IL-22 regulated AMPs, such as Reg3β and Reg3γ (**Fig. 13d**). Additionally, stress resulted an impairment in the expression of other important host defence mechanisms, including the reactive oxygen and nitrogen species and phospholipase A2, that could correspondingly be restored by IL-22-fc administration (**Fig. 13d**). We also identified an IL-22 independent loss of key antimicrobial defences following stress, including lysozymes and the family of alpha defenses (**Fig. 13d**). In line with these findings, our RT-qPCR analysis confirmed the ability of IL-22 to normalize the inflammatory environment induced by stress, significantly reducing transcript expression of *IL-23*, *IL-17A*, *IFN-γ,* and *IL-6* (**Fig. 13e**). Further, our results highlight a dichotomy of IL-22-dependent antimicrobial pathways that are modulated following stress. Wherein stress induces a profound impairment of IL-22 dependent AMPs while contributing towards heightened expression of genes responsible for iron limitation and the onset of nutritional immunity (**Fig. 13f**). Accordingly, IL-22-fc administration was able to drive AMP expression (**Fig. 13f**), likely contributing to the restoration of host innate defenses and impairing the outgrowth of AIEC typically seen following stress.

To investigate the implications of psychological stress and IL-22-fc administration in a model of colitis, we treated AIEC-colonized mice with a moderately low dose of DSS (2%). Mice were administered IL-22-fc prior to stress and every second day following stress release. Whereas mice that were exposed to stress or DSS alone maintained their baseline weights, stress mice treated with DSS failed to recover to baseline levels and lost greater than 10% of their initial body weight (**Fig. 13g**). Interestingly, administration of IL-22-fc was able to correct DSS-induced illness and IL-22 treated mice lost less weight than untreated mice exposed to stress (**Fig. 13g**). These results suggest that IL-22 signalling is able to prevent intestinal damage and DSS-induced illness that is typically exacerbated by overnight restraint stress. Moreover, we wanted to determine the impact of IL-22-fc administration on the constituents of the microbiome following stress. Accordingly, to determine whether IL-22 administration prior to restraint stress can correct the expansion of *Enterobacteriaceae* in the ileum, the microbiome was profiled using 16s rRNA sequencing of the V3/V4 region. While psychological stress led to a dominance of *Enterobacteriaceae*, IL-22 administration prevented stress-induced *Enterobacteriaceae* expansion (**Fig. 13h**). Together, these findings identify a stress-induced defect in the IL-22 pathway and highlight an essential role of IL-22 in mediating host protection against CD-associated pathobionts and preventing dysbiosis.

Discussion

Despite the growing global burden of CD, frontline treatments fail to adequately combat the underlying causes of intestinal inflammation, and thus continue to have a high rate of non-responsiveness. As such, there is an urgent need to thoroughly investigate the driving factors of CD and understand both their individual and combinatorial influence on the manifestation and progression of intestinal inflammation. Indeed, the intestinal microbiome of CD patients are commonly associated with a reduction of microbial diversity and a dominance of pathobiont strains of AIEC. Accordingly, populations of AIEC have been shown to flourish understates of intestinal perturbation commonly associated with CD including antibiotic use[26], infection[23], Western diet[27], and genetic susceptibility[28]. However, the contribution of individual microbial factors to CD remains elusive and their actual contribution to disease etiology is largely unknown. Additionally, individuals with CD often report a relapse of symptoms following states of acute psychological stress. Although psychological stress is typically associated with immunosuppression[37], conflicting reports have suggested stress-induced inflammation can lead to modest shifts in the constituents of the microbiome[33]. Accordingly, understanding the stress-induced changes to the intestinal microenvironment will be important in both understanding the mechanistic contributions of psychological stress to inflammation and designing targeted therapeutics to prevent disease relapse. In this work, we leverage a well-characterized model of restraint stress in conjunction with AIEC colonization to investigate the influence of psychological stress on host protection against a CD-associated pathobiont. Here we show that psychological stress results in an

impairment of host control of the AIEC population, resulting in profound intestinal dysbiosis and inflammation. We attribute the creation of a permissive niche for AIEC expansion to an exaggerated nutritional immune response in combination with a glucocorticoid-driven loss of the protective IL-22 producing population of $CD45^+CD90^+$ lymphocytes. Importantly, restoration of AMP defense via exogenous IL-22 delivery prevented the stress-associated expansion of AIEC and reduced DSS-induced sickness. As such, this work provides exciting insight regarding the mechanism of stress-induced dysbiosis and provides foundational research for the restoration of appropriate levels of IL-22 and related AMP defenses in the treatment of CD.

Previous studies have identified an anti-inflammatory role for psychological stress and glucocorticoid mediated responses[37,41,118]. Indeed, we have found that in our model of restraint stress, glucocorticoid signalling resulted in profound apoptosis of the $CD45^+CD90^+$ population of lymphocytes and an increase in production of IL-10. However, despite global lymphocytic depletion, we have found that stress resulted in a largely inflammatory profile. Accordingly, pathobiont strains of AIEC thrived under the resultant state of intestinal inflammation, driving the microbiome to closely mirror the dysbiotic state seen within inflamed regions of CD patients. Interestingly, in line with its established role as a pathobiont, the presence of AIEC was seen to exacerbate inflammatory signalling within the gut, in which the ability of AIEC to closely adhere to the intestinal epithelium likely contributed to an enhanced inflammatory immune response. Indeed, mice colonized with AIEC exhibited heightened pro-inflammatory

cytokine transcript expression and an impairment of intestinal barrier function. As such, it is likely that psychological stress facilitates the production of a positive feedback loop, such that pro-inflammatory mediated epithelial damage and bacterial dissemination fosters an environment favourable for the outgrowth of AIEC, further potentiating intestinal inflammation and damage. Our work highlights that in the presence of pathobiont strains of bacteria, who are particularly well equipt to thrive under states of inflammation, stress induced defects in the mucosal barrier create a homeostatic tipping point, favoring intestinal inflammation.

Interestingly, we note an essential role for the induction of nutritional immunity and iron limitation in the establishment of a permissive niche for AIEC expansion following psychological stress. Under homeostatic conditions, iron is typically transported complexed with heme and is thus a common target of iron acquisition for bacterial species[108,119]. As such in response to bacterial infection, the host sequesters free heme in pathophysiological settings[105,120]. Accordingly, we found that both HPX and haptoglobin were upregulated in stress, potentially limiting iron availability to bacterial species[103,105,121]. Further, in addition to sequestration of free heme, the host quickly acts to limit nutrients essential to bacterial survival and replication through the rapid deployment of Lcn2 and Calprotectin[122,123]. Accordingly, in response to selective pressure, *Enterobacteriaceae* have evolved alternative mechanisms to acquire metal ions under states of immune mediated iron restriction. Indeed, we have found that in response to stress-induced states of inflammation and dysbiosis, there is a robust recruitment of

$CD45^+CDllb^+Gr1^+$ neutrophil-like cells, major producers of the Lcn2 mediated nutritional immune response. However, similar to *Salmonella*, AIEC are equipt with an array of alternative siderophores in order to acquire the limited amounts of available iron and evade the Lcn2-mediated sequestration of enterobactin. As such, AIEC is provided a distinct competitive advantage over commensal strains of *E. coli* in the stress-exposed intestinal microenvironment. Further, AIEC is also enriched for other pathways that can be used to exploit states of nutrient limitation and quickly become a dominant member of the intestinal microbiome[86,107,124]. For example, AIEC has acquired the ZnuABC transporter, a high affinity iron transporter. Interestingly, *Salmonella* similarly express the ZnuABC transporter, allowing *Salmonella* to thrive under calprotectin-derived states of nutritional immunity[123]. In addition to a profound induction of Lcn2, our model of restraint stress results in a robust enrichment for the provision of zinc sequestration through the production of calprotectin. As such, it is likely that the ability of AIEC to acquire zinc despite nutritional immune induction similarly allows AIEC to thrive in the inflamed gut. These findings suggest that the ability of AIEC to evade host nutritional immune defense provides AIEC a distinct competitive advantage following psychological stress and provides mechanistic insight regarding stress-induced intestinal dysbiosis.

Interestingly, despite a profound outgrowth of AIEC and resulting microbial dysbiosis, there is a failure to launch a corresponding IL-22 mediated antimicrobial response. We have determined that the glucocorticoid-mediated response to restraint stress results in a consequential loss of the IL-22 producing subset of $CD45^+CD90^+$ lymphocytes. Further,

the loss of IL-22 signalling was determined to be crucial in the resultant expansion of AIEC characterized by stress. Indeed, restoration of IL-22 signalling was able to restore the host AMP response against intestinal dysbiosis and prevented the exaggerated DSS-induced sickness that was associated with psychological stress. Despite the maintenance of a heightened nutritional immune response, the provision of IL-22 removed the competitive advantage provided to AIEC understates of stress, likely due to a normalization of intestinal inflammation and restoration of AMP signalling. These findings identify a disconnect between cues of microbial dysbiosis and the corresponding induction of an IL-22 mediated immune response in the context of psychological stress and position glucocorticoids as the mechanistic link between psychological stress and IL-22 impairment. Recent clinical studies are evaluating the use of IL-22-Fc in the treatment of IBD patients (Clinical Trial: NCT03558152, NCT02749630), highlighting the therapeutic potential of IL-22 driven mucosal protection.

Interestingly, while most pathogenic insults to the gut drive a synchronized induction of both nutritional and mucosal immunity, psychological stress results in an uncoupling of these responses. Specifically, we demonstrate that the induction of nutritional immunity, as well as a loss of IL-22 mediated host defense are simultaneously required for the provision of a favourable niche for AIEC expansion. While AIEC is well equipt to evade a nutritional host defence and is provided a competitive advantage in the stress exposed gut, in the absence of the glucocorticoid mediated loss of IL-22 producing lymphocytes, AIEC forfeits its stress-derived competitive advantage. These findings provide novel

insight into the mechanism by which stress alters the intestinal microbiome and the ability of CD-associated pathobionts to exploit the resultant environment.

Limitations

Despite the exciting implications of our completed study, there are inherent limitations due to the study design. To begin, our model of restraint stress represents a highly severe stress event and is not representative of the acute stressors faced by CD patients in their everyday life. As such, although the results provide novel mechanistic understanding of the functional consequence of glucocorticoid signalling on host defense, these findings cannot be easily extended to explain the mechanism causing disease relapse in CD patients. To mirror lifestyle stress events more accurately, a chronic variable model of stress could be employed. Through this model, mice would be exposed to a variety of stressors, including cold exposure, prey exposure, short-term restraint, and cage shakes. In doing so, we would be able to investigate the immunological consequence of chronic stress exposure in a manner more similar to what is faced by the patient population. Additionally, in order to be consistent with published literature investigating susceptibility to colitis, we employed a DSS-model of colitis. However, we noted that overnight psychological stress most greatly sensitizes the ileum to the expansion of AIEC. To rectify this apparent disconnect, an ileitis model could be employed to investigate the influence of the stress induced expansion of AIEC on intestinal damage and inflammation.

As with all preclinical research, there is the inherent limitation that mouse models do not completely represent the biological processes of humans. Additionally, while all initial experiments were confirmed in female mice, all mechanistic findings were completed in male mice. Male mice were selected in this work as the glucocorticoid receptor antagonist RU486, can have off target effects on the progesterone receptor. Indeed, such experiments could be confirmed in female models to confirm an absence of a gender bias in our findings and ensure broad applicability.

Future Directions

This work presents exciting insight regarding the mechanisms by which psychological stress can sensitize the intestinal environment to bacterial dysbiosis and DSS-associated sickness. Such findings present interesting questions regarding the long-term functional consequences of stress in the context of susceptibility to colitis. Indeed, follow up work is required to understand the recovery of the lymphocytic population following global immune depletion mediated by glucocorticoid signalling. It will be interesting to determine the timescale of immune recovery within the gut following stress exposure and the resulting influence on host control of AIEC. Further, the proportions of T cell subsets in the recovered immune population can result in an immunological bias towards the creation of a pro-inflammatory or tolerogenic state. As such, the rebound of the lymphocytic population following stress can greatly influence susceptibility to the development of colitis. For example, a bias of pro-inflammatory T_H17 cells following recovery from overnight stress could contribute to the worsening of DSS-induced

sickness in our model of psychological stress and as such, will be important in fully delineating the mechanism by which stress can drive symptoms of colitis. Additionally, follow up work is needed to determine the combinatorial impact of psychological stress and other risk factors associated with CD. Indeed, we have shown that prior exposure to stress sensitizes mice to models of DSS-induced colitis. However, investigating alterations in inflammation and homeostasis following stress in the context of various risk factors including high fat diet, genetic susceptibility, and antibiotic use would provide a more holistic view of the biological underpinnings of CD. As a multifactorial disease, the ability to investigate the synergistic effects of various risk factors on disease progression will be essential in gaining an increasingly nuanced understanding of CD and designing improved therapeutic options for patients.

In addition to the long-term consequences of stress exposure, additional work is needed to understand the neuro-immune changes occurring within the gut. Indeed, previous reports have identified that the initiation of the HPA cascade and the release of CRF can lead to mast cell degranulation and increased intestinal permeability[60,61]. The initiating event of barrier breach and the resulting inflammation has yet to be determined in our model of restraint stress. Indeed, understanding the underlying factors leading to intestinal inflammation and disease relapse will be important in understanding the clinical consequences of stress and designing appropriate interventions. Further, studies have identified reciprocal regulation of IL-22 and the cytokine IL-18, whereby both can contribute to the increased production of AMPs and mucosal immunity[89,125]. Future work

investigating the influence of psychological stress on other immune pathways important for the maintenance of intestinal homeostasis will provide a deeper understanding of the mechanisms by which psychological stress can impair host defense against AIEC. Together, this research has the potential to extend our understanding of the functional consequences of the variety of signalling cascades engaged in response to psychological stress and leverage these findings to mitigate inflammation and safeguard intestinal homeostasis.

Conclusion

In summary, we have determined a role for psychological stress in the creation of a supportive niche for the unregulated expansion of a CD-associated strain of AIEC. We have identified the induction of nutritional immunity in conjunction with glucocorticoid-driven immune attrition as two necessary drivers of AIEC outgrowth following overnight restraint. Indeed, restoration of host mucosal immunity via exogenous IL-22-Fc delivery prevented AIEC expansion and corrected the DSS-induced sickness associated with stress. These findings extend our mechanistic understanding of the immunological consequences of psychological stress on host protection, providing novel insight regarding the association of stress and CD-symptom relapse. Understanding the various biological underpinnings of CD and their combinatorial effect on intestinal inflammation will play a critical role in our continued efforts to design targeted therapies to manage and address the underlying causes of CD.

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 set by the Canadian Council of Animal Care.

Animal infections and treatments

Six-to-eight-week-old C57BL/6N mice were purchased from Charles River Laboratories (QC, CAN). All mice were 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, mice were treated with 20 mg of streptomycin (Sigma-Aldrich, ON, CAN) by oral gavage. Mice were infected with 2×10^9 colony forming units (CFU) of AIEC strain NRG857c[126] in 0.1 ml sterile phosphate buffer saline (PBS). NRG857c was routinely grown shaking in Luria broth (LB, Sigma-Aldrich) with chloramphenicol (34 μg/mL) and ampicillin (200 μg/mL). NRG857c Δ*iroB* was grown as described above, with the addition of gentamicin (20 μg/mL). RU486 (Sigma-Aldrich) was dissolved in DMSO and delivered one hour prior to stress at a dose of 50 mg/kg, control mice were given an equivalent volume of DMSO. αCD90 treated mice were treated every other day intraperitoneally (i.p.) with 200 μg/mouse αCD90 (clone 53 2.1, BioXcell), starting on the day of infection. In some cases, LPS was administered i.p. at 0.25 mg/kg. IL-22 was blocked using 150 μg anti-IL-22 antibody (8E11; Genentech) dissolved in PBS delivered i.p. daily. IL-22-Fc (PRO312045; Genentech) was delivered to mice at a dose of 150 μg/mouse dissolved in PBS delivered i.p.. A matched IgG control

was given to control mice. For long-term anti-IL-22 treatment a dose of 150 µg/mouse was administered i.p. every other day for 11 days. All experiments were composed of 4 mice per group and the total number of mice used are represented in the figure legends. Male mice were used in order to minimize the confounding effects of the female estrus cycle on RU486 treatment. All preliminary results were confirmed in female mice.

Restraint stress

Stress exposed mice were placed in well ventilated 50 mL conical tubes (20 holes per tube) for 16 h during their dark cycle. Matched control mice were deprived of food and water for 16 h [112,113]. For tissue CFU enumeration, mice were immediately sacrificed, and samples were collected in sterile PBS. In some experiments, mice were allowed to recover, and fecal samples were collected at 6 and 24 h following restraint stress. In repeated stress experiments, mice were subjected to weekly overnight restraint stress and allowed to recover, with fecal samples collected prior to and at 6 and 24 h post-stress. In one experiment, mice were sacrificed after 4 h of stress and samples were taken for RT-qPCR analysis.

Bacterial enumeration

Fecal or tissue samples were collected in 1 mL sterile PBS. The small intestine was divided into four equal segments as previously described[127]. Segment 1 denotes the segment closest to the stomach and segment 4 closest to the cecum. Segment 4 represents the ileal region of the small intestine and is used interchangeably throughout. Whole

tissue from four segments from the small intestine, as well as the cecum and colon, were collected at the time of sacrifice and placed in 1 mL sterile PBS and homogenized using a Mixer Mill homogenizer. Homogenized samples were serially diluted and plated on LB agar supplemented with ampicillin (200 µg/mL) and chloramphenicol (34 µg/mL). Liver samples were processed similarly and plated undiluted on un-supplemented LB agar for the enumeration of total viable bacteria.

mRNA assessment by reverse-transcriptase quantitative PCR

Tissue samples were taken from the distal 1 cm of ileum (Segment 4) and placed in TRIzol (Invitrogen, ON, CAN). Total RNA was extracted and converted to complementary DNA (cDNA) using a one component cDNA SuperMix (Quanta Biosciences, MA, USA). Quantitative PCR was performed on a Lightcycler 480 (Roche, QC, CAN), using SYBR green SuperMix (Quanta Biosciences). All primers used in the study are listed in Table 1. The cycling conditions were 95 °C for 5 min and 50 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 20 s. Gene expression was evaluated using the $2^{-\Delta\Delta CT}$ method and samples were normalized to the housekeeping gene, *RPLP0*, and expressed relative to the control starved mice.

Quantification of biomolecules

Liver LPS levels were enumerated using a Pierce™ Chromogenic Endotoxin Quant Kit (Thermo Fisher Scientific, MD, USA). Values were represented as endotoxin units (EU). Mouse Lcn2/NGAL and S100a8 within tissue homogenates were enumerated using a

DuoSet ELISA (R&D Systems, MN, USA) as per the manufacturer's protocol. Numerical values were determined using an 8-point standard curve of known concentrations. Mouse IL-22 within the ileal explant supernatant was enumerated using an ELISA (ThermoFisher) as per the manufacturer's protocol. Numerical values were calculated using an 8-point standard curve of known concentrations. Mouse corticosterone within the serum was enumerated using a Corticosterone Parameter Assay Kit (R&D Systems) as per the manufacturer's protocol. Numerical values were determined using a 6-point standard curve of known concentrations.

16S rRNA sequencing

Luminal contents were scraped from the various intestinal segments taken from control mice (*ad libitum* food/water), control (food/water restricted), stressed, and stressed with IL-22-Fc-treated mice and placed in a sterile collection tube containing $NaPO_4$ and guanidine thiocyanate/EDTA. All mice arrived at animal facilities in the same shipping crate, were caged in the same bedding, and treated under the same conditions prior to stress. Samples were processed by the Farncombe Metagenomics Facility (McMaster University). 16s rRNA V3/V4 regions were sequenced using an Illumina sequencer. Taxonomy was assigned to the output sequences using the Silva reference dataset (v 132) using the R package DADA2. Taxonomical plots were created using the R packages phyloseq and phytools and plotted using ggplot2. Relative proportional change was calculated based on the frequency of bacterial species present in the starved control mice compared to overnight stressed mice. Values were plotted as $\log_{10}$ change in abundance.

Community diversity was assessed by calculating the Shannon diversity index using the R package, vegan. The factoextra package was used to calculate the eigenvectors for Principal Component Analysis and plotted using ggplot2.

RNA sequencing of bulk intestinal tissue.

Tissue was isolated from the distal 1 cm of ileum and placed in TRIzol. RNA was extracted and frozen in DEPC water prior to shipment to Genewiz for RNA sequencing. Ribosomal RNA was removed using a Ribo-Zero Gold Kit (Illumina, San Diego, CA, USA), and the sequencing library prepared using a NEBNext Ultra RNA Library Prep Kit from Illumina. Sequencing analysis was done using an Illumina HiSeq using a 2x150bp paired end configuration, single index, per lane. One mismatch was allowed for index sequence identification. Transcripts were quantified using Salmon[128] and differential expression levels were evaluated using the R package DeSeq2[129]. Heatmaps were generated using the R package pHeatmap[130].

Intestinal permeability measurements

Ussing chambers were used to evaluate the permeability of ileum sections *ex vivo* following overnight starvation or stress. Paracellular permeability was assessed using 6 μCi/mL of ^{51}Cr-EDTA (type II; Perkin Elmer) probes. ^{51}Cr-EDTA was measured in a liquid scintillation counter and reported as percent recovery/cm^2/h.

Dextran sodium sulfate (DSS)-induced colitis

Mice were started on 2% DSS in drinking water *ad libitum* (40,000 MW– 50,000 MW, Thermo Scientific) on day 3 post-infection with NRG857c and mice were stressed for 16 h on day 5. Mice were switched back to normal drinking water on day 8 or 9. Body weight was recorded daily. In cases where mice were treated with IL-22-Fc (150µg prior to stress, 50µg every two days following stress), matched groups received an equivalent volume of IgG.

Construction of mutants

Lambda Red recombination[131] was used to delete the *iroB* gene with modified plasmids that allowed for selection with gentamicin. Primer sequences used for mutant generation and screening are listed in Table 2. In brief, primer pairs containing extensions complementary to 100 bp in-frame regions at the 5' and 3' ends of each gene of interest were used to PCR amplify the gentamicin resistance cassette from pCDF_GmFrt and generate knockout constructs. PCR products were purified and transformed into NRG857c harboring pKD46_km. Transformants were selected on LB-agar containing 20 µg/mL gentamicin and successful deletion of genes was confirmed through PCR and sequencing (Genewiz, South Plainfield, NJ).

Competitive infections of mice

Strains of wildtype or mutant NRG857c were grown overnight in LB with selection as described above. For competitive infections, mice were treated with streptomycin (20 mg)

one day before infection and infected with 2×10^9 CFU of an equal mixture of competing strains. Mice were exposed to overnight restraint stress or food/water deprivation on day 2 following infection. Bacterial CFU was enumerated within tissue samples as described above. In order to account for *in vivo* bottlenecks, the competitive index was normalized to the average ratio of wildtype to mutant AIEC within fecal samples taken at the time of stress.

Cell isolation and flow cytometry

Epithelial cell and lamina propria cell isolation - Ileum was extracted from control, stressed, and IL-22-treated, stressed mice at the time of sacrifice and placed in cold PBS. Feces was removed and samples were opened longitudinally before being cut into 1 cm segments and placed in 15 mL tubes containing 10 mL PBS. Samples were washed in PBS, placed in 10 mL of 5 mM EDTA and 2 mM DTT, and incubated at 37°C. Samples were rotated at 15 rpm in a hybridization oven for 30 mins and filtered through a 100 µM filter. Following epithelial cell isolation, cells were further digested for the isolation of lamina propria cells in 12.5 µg/mL Liberase TM (Sigma-Aldrich) and 1 mg/mL DNase I (Sigma-Aldrich) at 37°C. Control samples are comprised of 2 pooled mice and all stress samples are comprised of four pooled mice. Samples were rotated at 8 rpm in a hybridization oven for 10 mins and filtered through a 100 µM filter. Samples were enriched for mononuclear cells using a 40% Percoll gradient. Isolated cells were incubated with CD16/32 (Fc Block) for 10 min prior to staining. For epithelial cell analysis cells were stained for 30 min with CD45 - eflour450 (1:400), EpCam - APC

(1:400) in 2% FBS at 4°C. Following surface staining, cells were washed, fixed, and permeabilized using a *Foxp3* transcription factor staining buffer (Thermofisher) for 20 min at room temperature. Following fixation, cells were stained for 30 min with 5 μL of pSTAT-3-Pe Cyanine 7 (Thermofisher), washed and resuspended in 2% FBS/PBS. Isolated lamina propria cells were stained for 30 minutes with a cocktail of lineage exclusion markers (Ter119, Gr1, CD3, CD11b, B220) – FITC (1:400), CD90 – APC eflour 780 (1:600), CD45 – eflour 450 (1:600), TCRβ - SuperBright785 (1:600), CD4 – APC eflour 780 (1:400), CD8 – Alexa Fluor 532 (1:400) for lymphocyte analysis, or CD45 – PercpCy5.5 (1:600), CD11b – eflour 605 (1:800), Gr1– FITC (1:800) for neutrophil-like cells. Following surface staining, cells were washed, fixed, and permeabilized using a *Foxp3* transcription factor staining buffer (Thermofisher) for 20 min at room temperature. Cells were stained intracellularly for IL-22 – PercpCy5.5 (5 μL/test), rorγt – APC (1:50), GR – PE Cyanine 7 (1:20) for 30 min in 1x Perm/fix buffer at room temperature. For Annexin V staining isolated lamina propria cells were washed with PBS and Annexin binding buffer (ThermoFisher) and stained for 25 min at 37°C with CD45 – PercpCy5.5 (1:600), CD90 – PE Cyanine 7 (1:600), Annexin V – APC (5 μL/test). Cells were washed and samples were evaluated using a LSRII flow cytometer and analysis was performed using FlowJo (v9, BD Biosciences).

Explants

Ileum was extracted from mice at the time of sacrifice and the fecal content was cleared and washed with cold PBS. After washing, 1 cm sections of ileum were treated with 100

μg/mL of gentamicin for 1 h, washed and cultured for 24 h in complete RPMI (10% FBS, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES) with or without rIL-23 (20 ng/mL) and supernatants were collected after 24 h.

Immunohistochemistry

Mouse ileum were fixed using 10% formalin following stress, paraffin embedded, and stained for Gr1 at the Histology Services (McMaster University). IHC quantification represents an average of four sections per sample.

Statistical analysis

A Mann-Whitney test was applied to experimental data where only two groups were present. A standard one or two-way ANOVA was used for comparisons of three or more groups depending on the experimental design. Correlation analysis was done using a Spearman correlation analysis. Values were considered statistically significant for $p < 0.05$. Statistical analysis was performed using Prism GraphPad (v8).

Tables and Figures

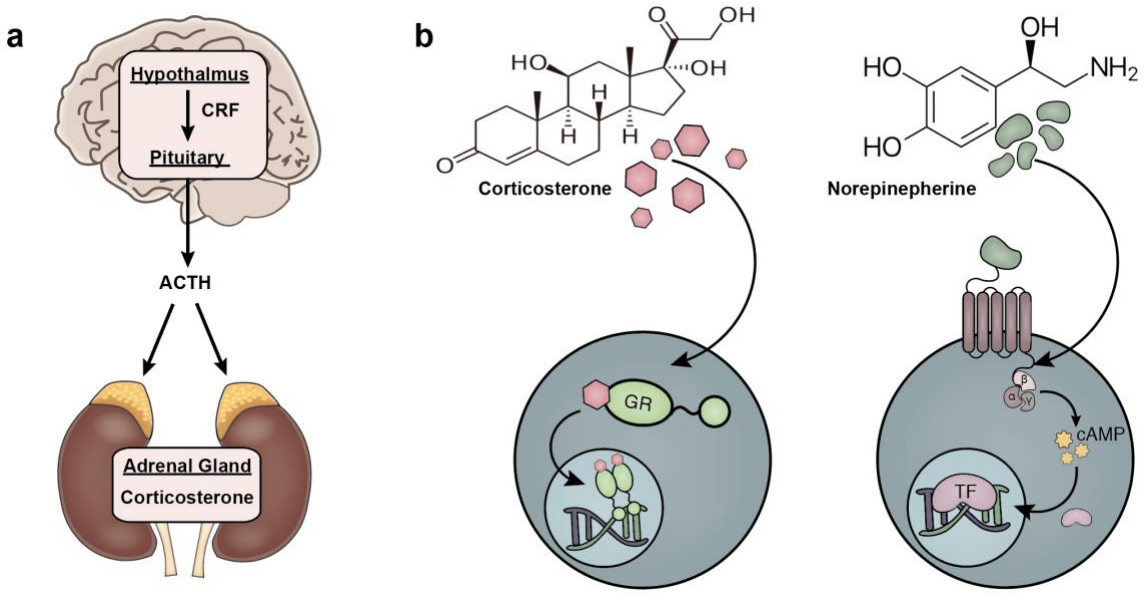


Figure 1: Stress induced signalling pathways. a, Diagrammatic representation of the HPA cascade. **b,** Representative signalling cascade of the two major arms of the stress response. Corticosterone signals intracellularly to the glucocorticoid receptor to alter transcription. Norepinephrine signals to a G-coupled protein receptor to alter cytoplasmic levels of cAMP and alter transcription.

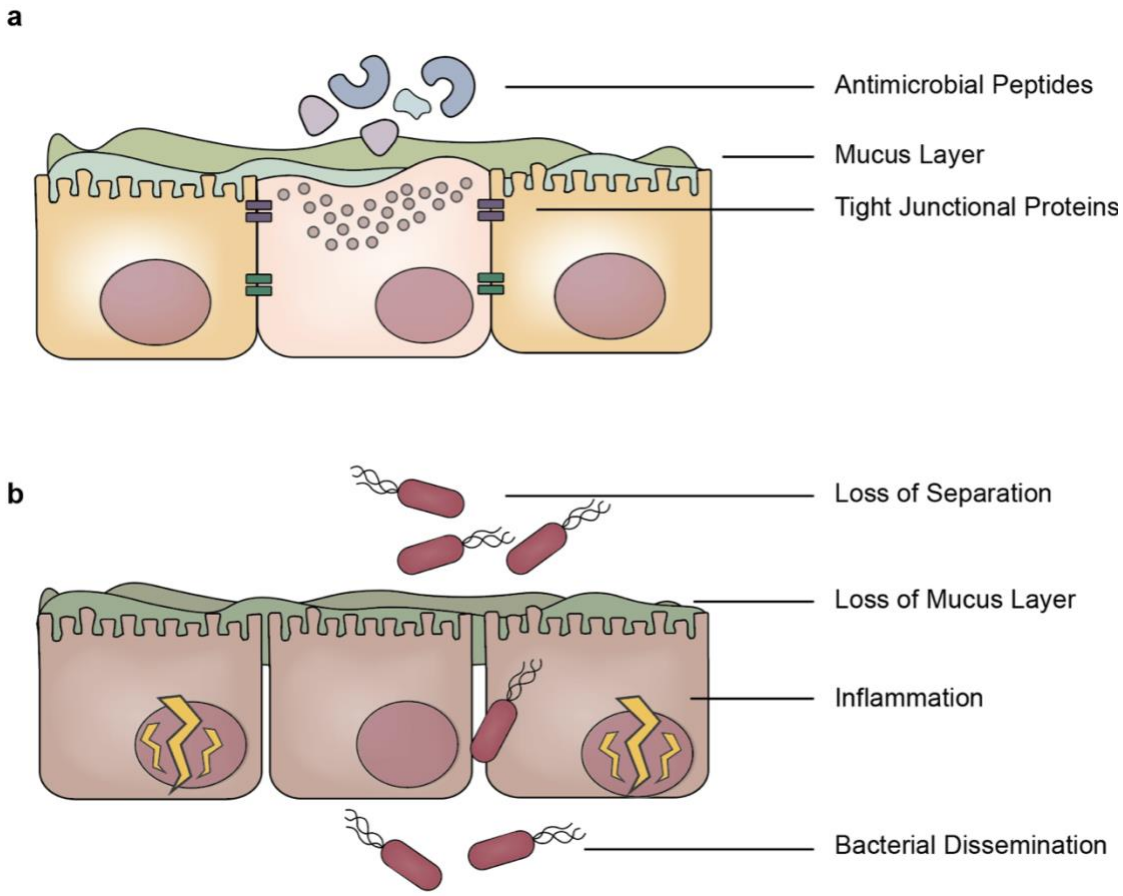


Figure 2: Innate host defenses of the intestine. a, Diagrammatic representation of the various innate defences under states of homeostasis. **b,** Diagrammatic representation of the consequence of inflammation on intestinal innate defenses.

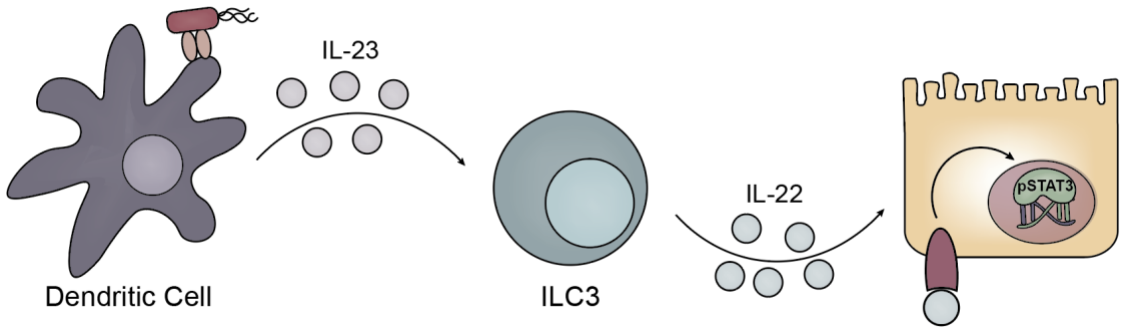


Figure 3: IL-22 signalling by ILC3s is induced in response to microbial ligands.

Following the engagement of the TLR4 receptor, innate immune subsets such as dendritic cells and macrophages release the cytokine IL-23. Upstream of IL-22 production, IL-23 will engage its receptor on the surface of an IL-22 producing cell including ILC3s. IL-22 signals through a dimeric IL-10Rβ : IL-22R1 receptor to initiate the phosphorylation of STAT-3. STAT-3 phosphorylation can modulate the expression of downstream IL-22 dependent genes to respond to the initiating stimuli.

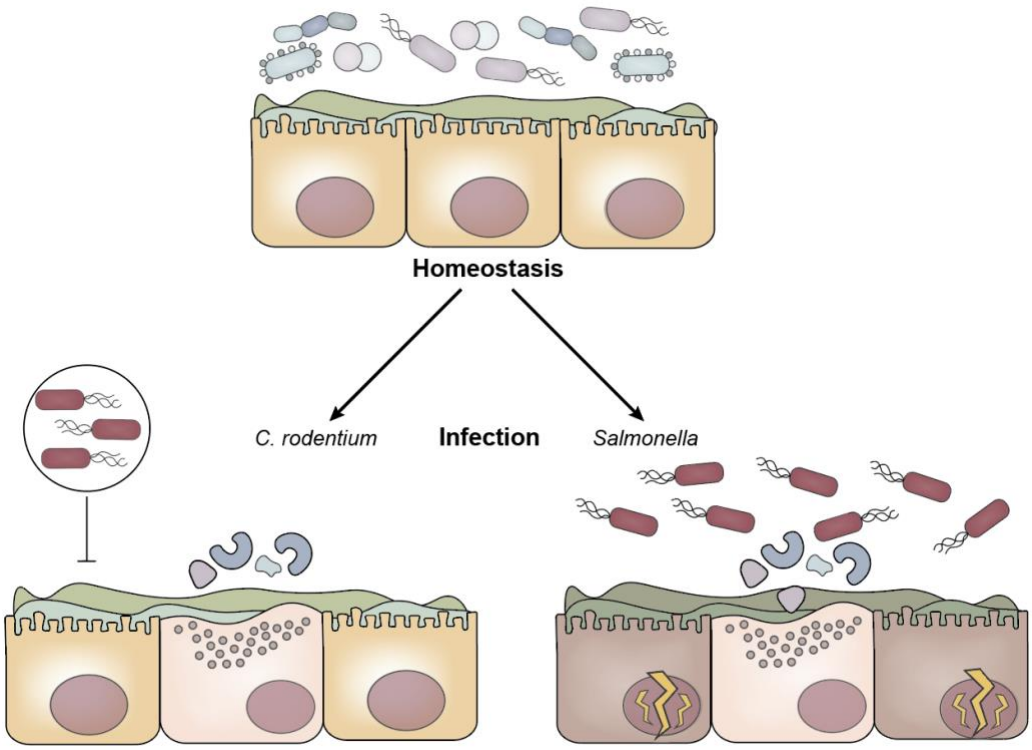


Figure 4: IL-22 signalling can have diverging consequences in response to bacterial infection. While IL-22 is commonly associated with intestinal homeostasis, the context of the IL-22 response is decisive in infection resolution. In the context of *C. rodentium* infection, IL-22 robustly induces the production of AMPs and is able to prevent bacterial colonization and damage. However, in *Salmonella* infection models, a robust IL-22 response was seen to be detrimental in host protection. *Salmonella* can evade IL-22 dependent defences such as iron limitation through the deployment of alternative siderophores. As such, IL-22 allows *Salmonella* to outcompete commensal microbes and thrive in the inflamed gut. These microbes highlight the contextual nature of IL-22 driven responses in host protection.

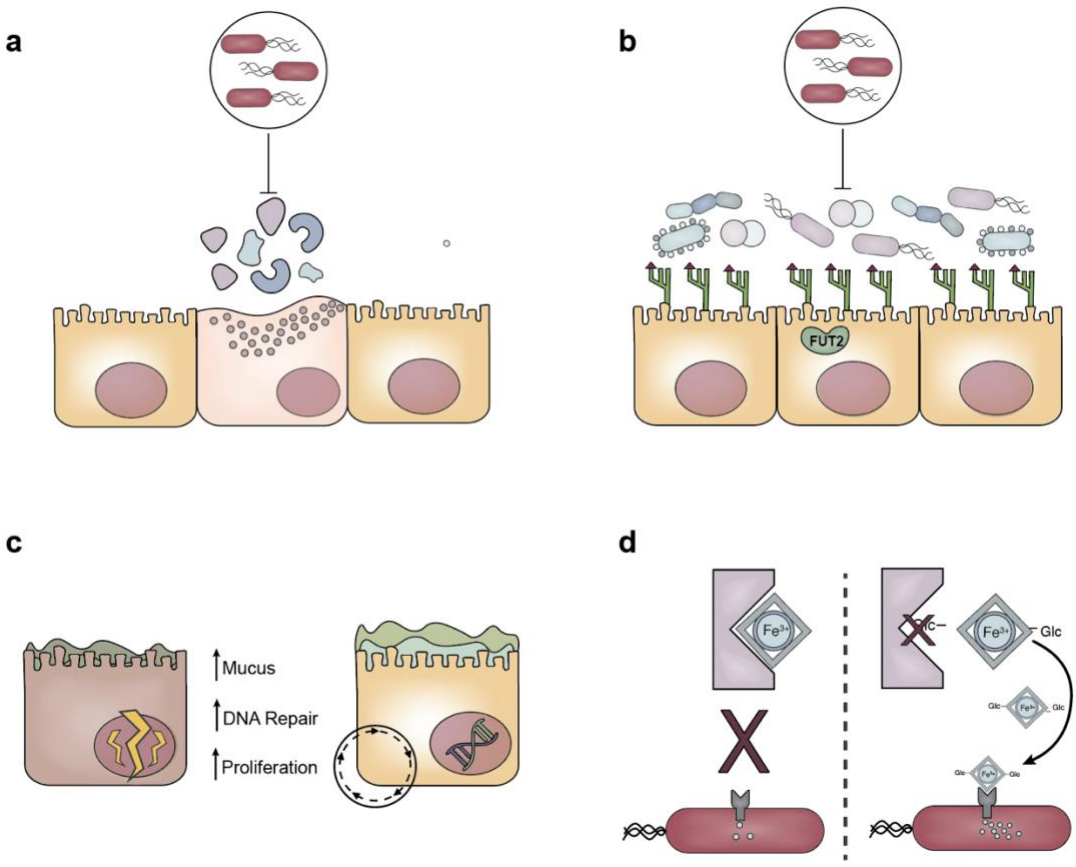


Figure 5: IL-22 signalling functions to maintain intestinal homeostasis through a variety of mechanisms. a, In bacterial dysbiosis, IL-22 can robustly induce the expression of AMPs by epithelial cells in order to limit their colonization. Further, basal AMP production by IL-22 permits the establishment of the host microbiome and provides colonization resistance. **b,** IL-22 signalling in response to microbial ligands favours the commensal population through the addition of fucosyl and glycosl residues to epithelial cells, further ensuring the maintance of a colonization barrier. **c,** IL-22 is also an important regulator of epithelial repair, proving to be essential in efforts to increase mucus production, DNA repair, and epithelial proliferation. As such, IL-22 is important

in restoring the intestine following damage in models of colitis. **d,** In the event of infection, IL-22 can induce a state of nutritional immunity in which IL-22 driven Lcn2 production acts to sequester bacterial derived siderophores to prevent their iron consumption. However, evolved species of bacteria such as *Salmonella* have aquired alternative mechanisms of iron aquistion, allowing them to thrive following an IL-22 driven response.

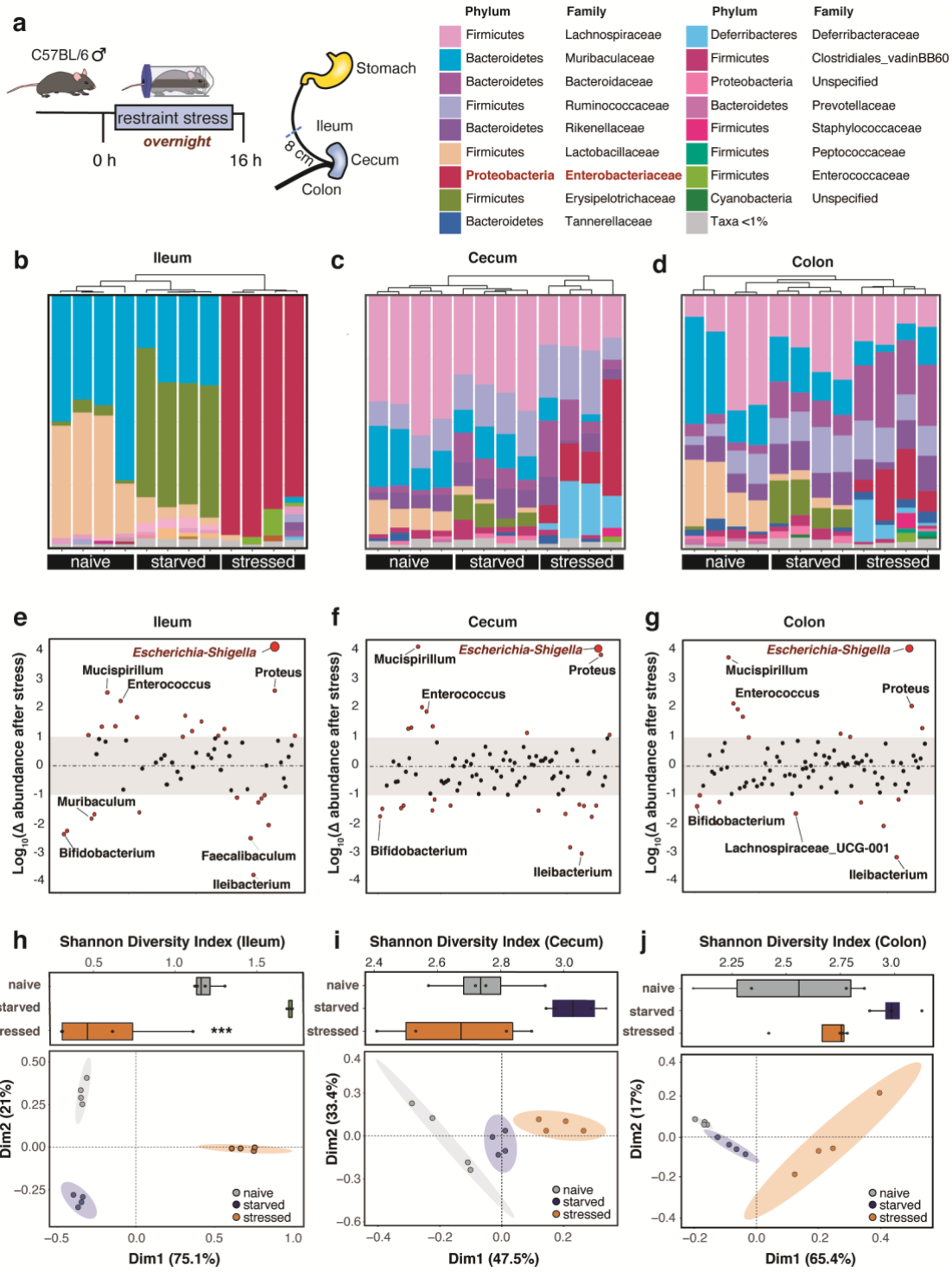
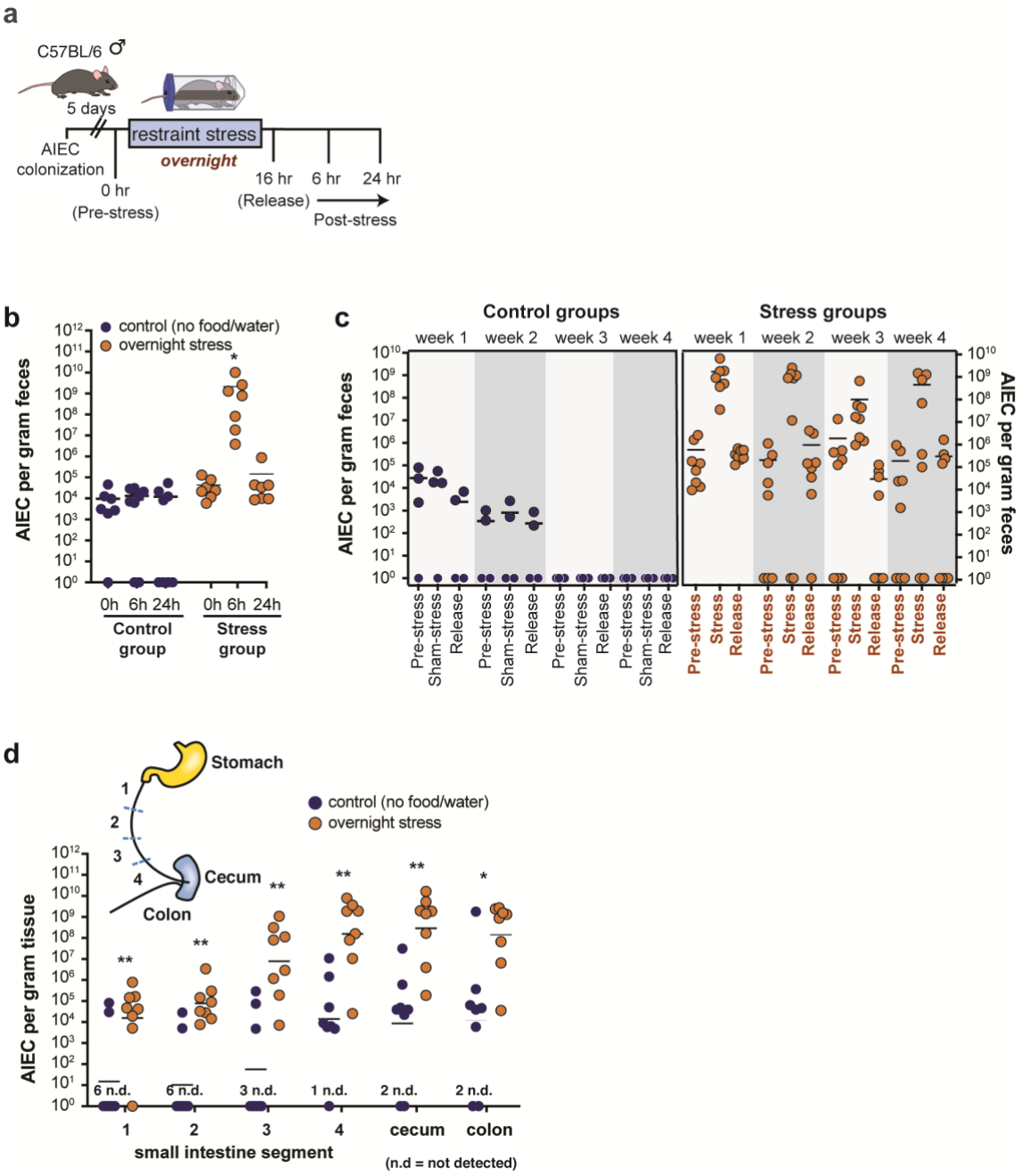
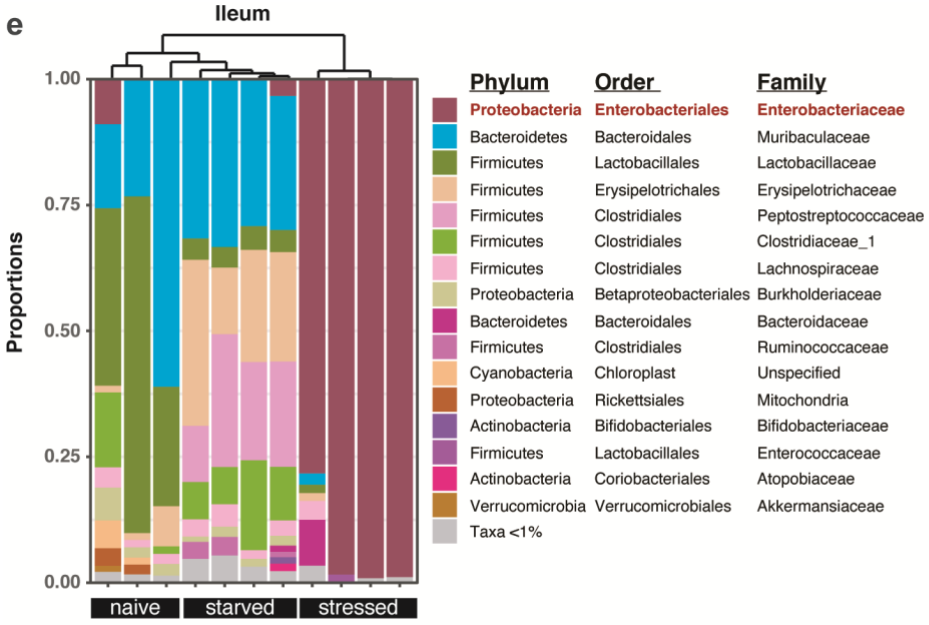

a
C57BL/6♂
restraint stress
overnight
0 h
16 h
Stomach
Ileum
8 cm
Cecum
Colon
Phylum
Family
Firmicutes
Lachnospiraceae
Bacteroidetes
Muribaculaceae
Bacteroidetes
Bacteroidaceae
Firmicutes
Ruminococcaceae
Bacteroidetes
Rikenellaceae
Firmicutes
Lactobacillaceae
Proteobacteria
Enterobacteriaceae
Firmicutes
Erysipelotrichaceae
Bacteroidetes
Tannerellaceae
Deferribacteres
Deferribacteraceae
Firmicutes
Clostridiales_vadinBB60
Proteobacteria
Unspecified
Bacteroidetes
Prevotellaceae
Firmicutes
Staphylococcaceae
Firmicutes
Peptococcaceae
Firmicutes
Enterococcaceae
Cyanobacteria
Unspecified
Taxa <1%
b
Ileum
c
Cecum
d
Colon
naive
starved
stressed
e
Ileum
f
Cecum
g
Colon
Log10(Δ abundance after stress)
Escherichia-Shigella
Mucispirillum
Enterococcus
Proteus
Muribaculum
Bifidobacterium
Faecalibaculum
Ileibacterium
Lachnospiraceae_UCG-001
h
Shannon Diversity Index (Ileum)
i
Shannon Diversity Index (Cecum)
j
Shannon Diversity Index (Colon)
***
Dim2 (21%)
Dim1 (75.1%)
Dim2 (33.4%)
Dim1 (47.5%)
Dim2 (17%)
Dim1 (65.4%)

Figure 6. Psychological stress promotes the expansion of ileal *Enterobacteriaceae*.

a, Schematic representation of the stress protocol and legend of bacterial phylum and family. Taxonomy plots of 16S rRNA sequencing of the ileal **b**, cecal **c**, or colonic **d,** contents of naive (n = 4), starved (food and water deprived, n = 4), and restraint-stress (n = 4) exposed mice. Analysis of the proportional change in species abundance seen following restraint stress in the ileum **e**, cecum **f**, or colon **g**. Change was calculated based on the relative proportional change of species abundance in stress mice, compared to the species abundance in the starved control. Shannon Diversity Index and Principal Component Analysis of ileal **h**, cecal **i**, or colonic **j,** 16S rRNA sequencing from naive (n = 4), starved (n = 4) and stress (n = 4) mice. PCA plots display a 95% confidence interval in the ileum, and 80% confidence interval in the cecum and colon. For Shannon Diversity Index, significance between starved and stressed mice was determined by one-way ANOVA. (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).


a
C57BL/6 ♂
5 days
restraint stress
overnight
AIEC colonization
0 hr (Pre-stress)
16 hr (Release)
6 hr
24 hr
Post-stress
b
control (no food/water)
overnight stress
AIEC per gram feces
0h 6h 24h
Control group
Stress group
c
Control groups
Stress groups
week 1
week 2
week 3
week 4
AIEC per gram feces
Pre-stress
Sham-stress
Release
Stress
d
Stomach
Cecum
Colon
control (no food/water)
overnight stress
AIEC per gram tissue
6 n.d.
3 n.d.
1 n.d.
2 n.d.
1
2
3
4
cecum
colon
small intestine segment
(n.d = not detected)


e
Ileum
Proportions
1.00
0.75
0.50
0.25
0.00
naive
starved
stressed
Phylum
Order
Family
Proteobacteria Enterobacteriales Enterobacteriaceae
Bacteroidetes Bacteroidales Muribaculaceae
Firmicutes Lactobacillales Lactobacillaceae
Firmicutes Erysipelotrichales Erysipelotrichaceae
Firmicutes Clostridiales Peptostreptococcaceae
Firmicutes Clostridiales Clostridiaceae_1
Firmicutes Clostridiales Lachnospiraceae
Proteobacteria Betaproteobacteriales Burkholderiaceae
Bacteroidetes Bacteroidales Bacteroidaceae
Firmicutes Clostridiales Ruminococcaceae
Cyanobacteria Chloroplast Unspecified
Proteobacteria Rickettsiales Mitochondria
Actinobacteria Bifidobacteriales Bifidobacteriaceae
Firmicutes Lactobacillales Enterococcaceae
Actinobacteria Coriobacteriales Atopobiaceae
Verrucomicrobia Verrucomicrobiales Akkermansiaceae
Taxa <1%

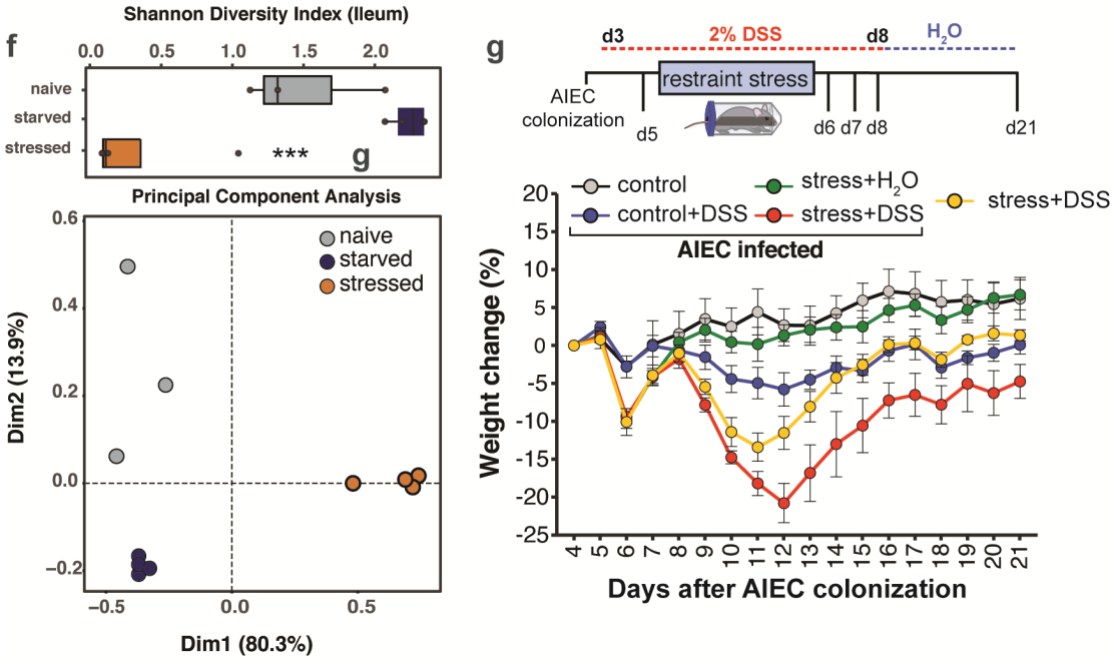
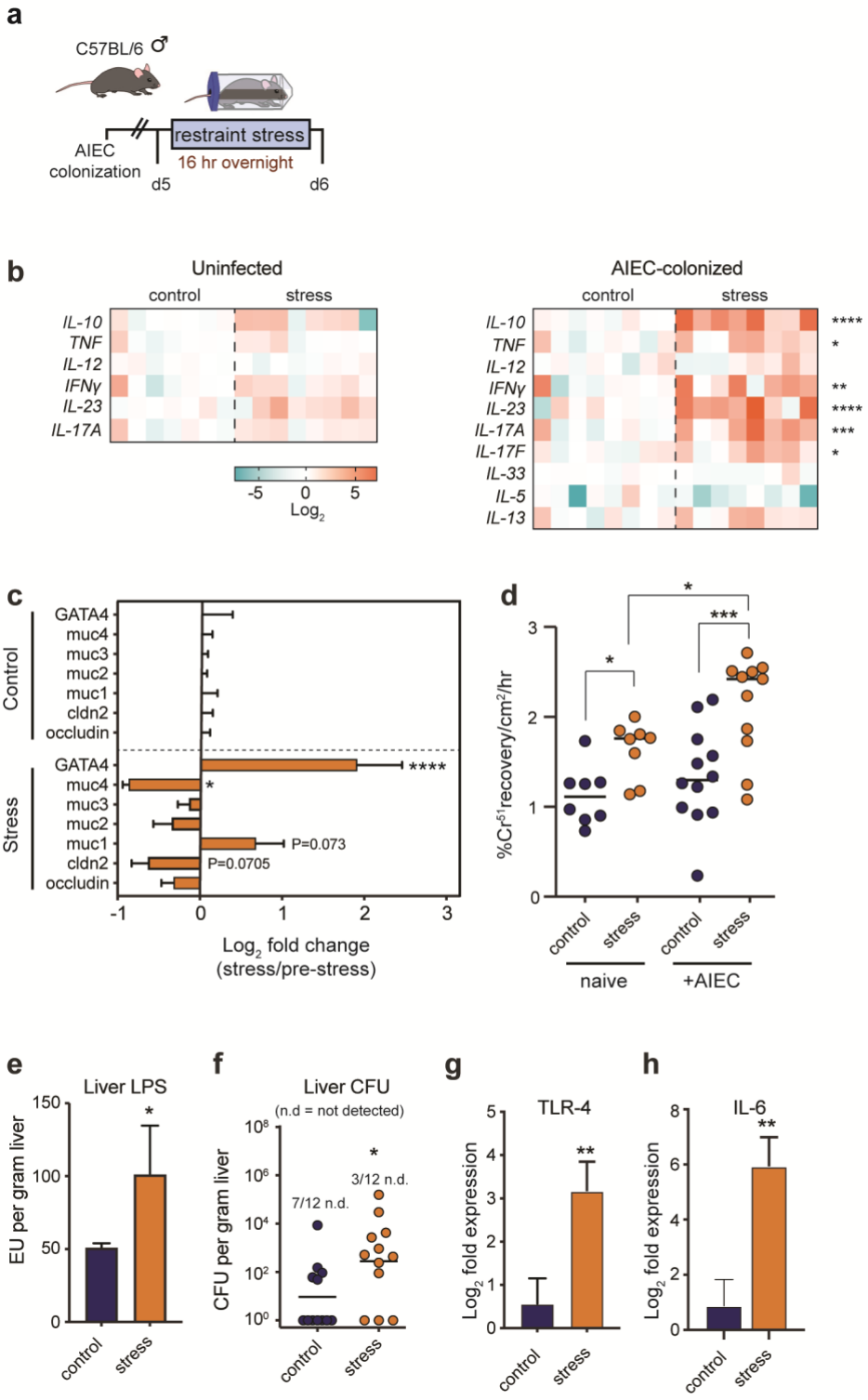

f
Shannon Diversity Index (Ileum)
0.0 0.5 1.0 1.5 2.0
naive
starved
stressed
***
g
Principal Component Analysis
Dim2 (13.9%)
0.6
0.4
0.2
0.0
−0.2
naive
starved
stressed
−0.5 0.0 0.5
Dim1 (80.3%)
g
d3
2% DSS
d8
H2O
AIEC colonization
d5
restraint stress
d6 d7 d8
d21
control
control+DSS
stress+H2O
stress+DSS
stress+DSS
AIEC infected
Weight change (%)
20
15
10
5
0
-5
-10
-15
-20
-25
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days after AIEC colonization

Figure 7. Psychological stress impairs host control of CD-associated AIEC.

a, Schematic representation of infection and stress protocol. **b**, AIEC fecal burdens collected from control (n = 8) and stress (n = 7) exposed mice at the time of stress (or overnight starvation), and at 6 h and 24 h post-stress. Stress exposed mice were compared to matched controls by a two-way ANOVA. **c**, Mice were colonized with AIEC and subjected to either overnight stress (n = 8) or starvation (n = 4) for 16 h weekly for four weeks. Fecal samples were collected at the time of stress (or overnight starvation), and at 6 h and 24 h post-stress. **d**, AIEC tissue burdens collected from the length of the intestinal tract from control (n = 8) and stress (n = 8) mice. The small intestine was divided into four 8 cm segments in which segment 4 is adjacent to the cecum. Stress exposed mice were compared to match controls by a Mann-Whitney test. **e**, Taxonomy plot of 16S rRNA sequencing of ileum in AIEC-colonized naive (n = 3), starved (food and water depleted, n = 4), and restraint-stress (n = 4) exposed mice. **f**, Shannon Diversity Index and Principal Component Analysis of ileal 16S rRNA sequencing from AIEC-colonized naive (n = 3), starved (n = 4) and stress (n = 4) mice. **g,** Schematic representation of DSS treatment schedule. The graph depicts weight change from the time of DSS initiation, (all groups are n = 4). (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). Error bars represent SEM and the line in CFU graphs indicates the geometric mean of the group.


a
C57BL/6 ♂
AIEC colonization
restraint stress
16 hr overnight
d5
d6
b
Uninfected
AIEC-colonized
control
stress
IL-10
TNF
IL-12
IFNγ
IL-23
IL-17A
IL-17F
IL-33
IL-5
IL-13
Log2
c
Control
Stress
GATA4
muc4
muc3
muc2
muc1
cldn2
occludin
P=0.073
P=0.0705
Log2 fold change (stress/pre-stress)
d
%Cr51 recovery/cm2/hr
naive
+AIEC
e
Liver LPS
EU per gram liver
f
Liver CFU
(n.d = not detected)
CFU per gram liver
7/12 n.d.
3/12 n.d.
g
TLR-4
Log2 fold expression
h
IL-6

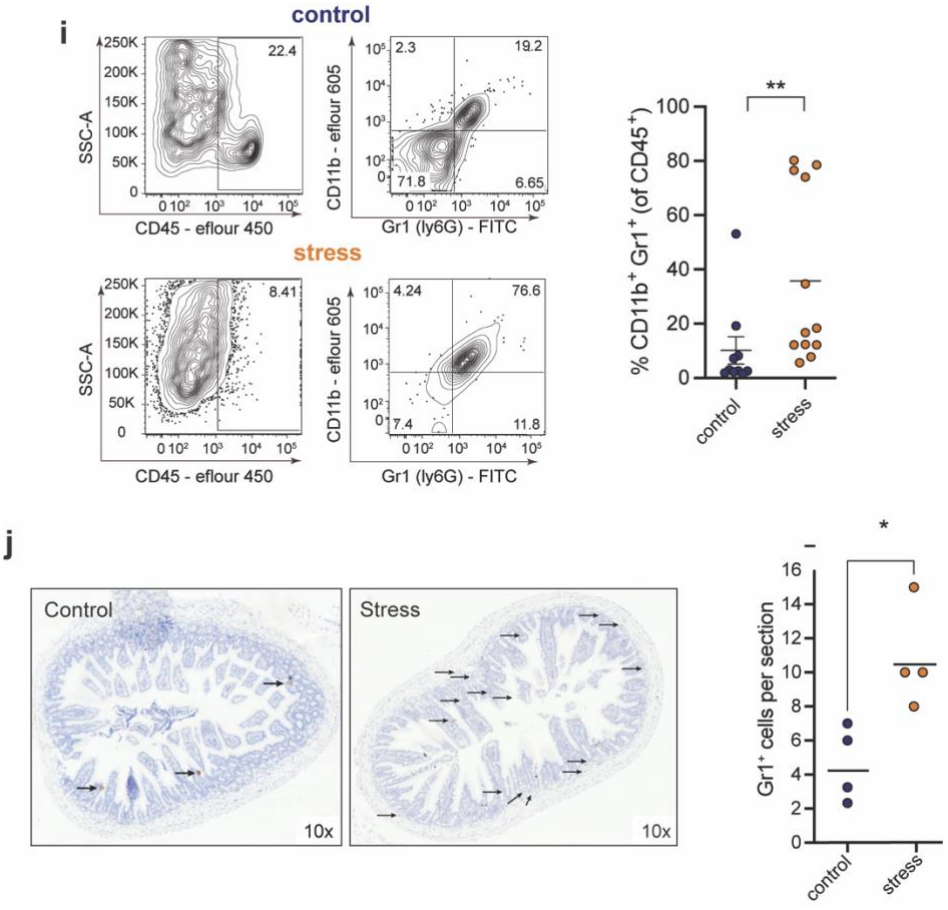
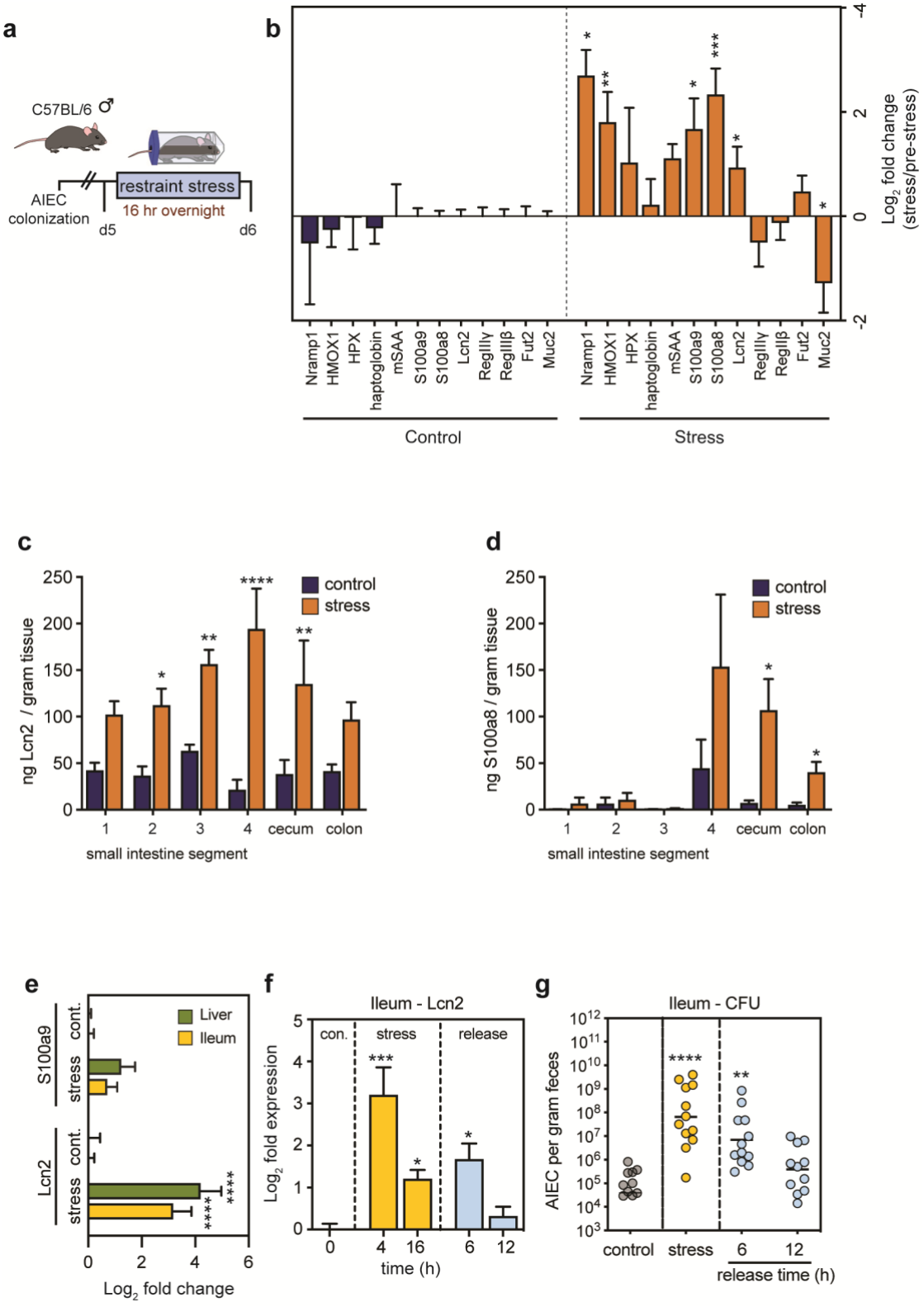

i
control
SSC-A
CD45 - eflour 450
22.4
CD11b - eflour 605
Gr1 (ly6G) - FITC
2.3
19.2
71.8
6.65
stress
8.41
4.24
76.6
7.4
11.8
% CD11b+ Gr1+ (of CD45+)
**
control
stress
j
Control
Stress
10x
Gr1+ cells per section
*

Figure 8. Exposure to acute psychological stress impairs ileal barrier function and exposes mice to invasive microbes. a, Schematic representation of infection and stress protocol. **b,** RT-qPCR analysis of cytokine expression of the ileum of naive starved (n = 7), naive stressed (n = 8), AIEC-colonized starved (n = 8), AIEC-colonized stressed (n = 8) samples. Significance was determined by a two-way ANOVA. **c,** RT-qPCR analysis of RNA expression for genes associated with barrier function in the ileum of AIEC-colonized starved (n = 12 - 20) and stress (n = 12 - 20) mice. Significance was determined by a two-way ANOVA. **d,** Paracellular permeability determined by chromium passage through a 1 cm ileal segment collected from naive (n = 8) or AIEC colonized (n = 12) mice subjected to starvation or restraint stress. Significance was determined by a two-way ANOVA. **e,** Liver LPS concentration, represented as Endotoxin Units per gram tissue, was quantified within AIEC-colonized starved (n = 4) and stressed (n = 4) mice. Significance was determined by Mann-Whitney. **f,** Bacterial burdens in the liver were enumerated in AIEC-colonized starved (n = 12) or stressed (n = 12) mice. Significance was determined by Mann-Whitney. **g,** RT-qPCR analysis of TLR4 RNA expression in the ileum of AIEC-colonized starved (n = 12) or stressed (n = 12) mice. Significance was determined by Mann-Whitney. **h,** RT-qPCR analysis of IL-6 RNA expression in the ileum of AIEC-colonized starved (n = 8) or stressed (n = 8) mice. Significance was determined by Mann-Whitney. **i,** Representative FACS plots of $CD45^+CD11b^+GR1^+$ cells. Percentage of $CD11b^+Gr1^+$ cells from the total population of $CD45^+$ cells isolated from ileal lamina propria cells collected from starved (n = 8) and stressed (n = 8) mice as determined by flow cytometry. Significance was determined by Mann-Whitney. **j,**

Number of $GR1^+$ cells as determined by immunohistochemistry. Significance was determined by Mann-Whitney. (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$). Error bars represent SEM and the line in CFU graphs indicates the geometric mean of the group.


a
C57BL/6 ♂
restraint stress
AIEC colonization
16 hr overnight
d5
d6
b
Log2 fold change (stress/pre-stress)
Nramp1
HMOX1
HPX
haptoglobin
mSAA
S100a9
S100a8
Lcn2
RegIIIγ
RegIIIβ
Fut2
Muc2
Control
Stress
c
ng Lcn2 / gram tissue
control
stress
small intestine segment
cecum
colon
d
ng S100a8 / gram tissue
e
S100a9
Lcn2
cont.
stress
Liver
Ileum
Log2 fold change
f
Ileum - Lcn2
Log2 fold expression
con.
stress
release
time (h)
g
Ileum - CFU
AIEC per gram feces
control
stress
release time (h)

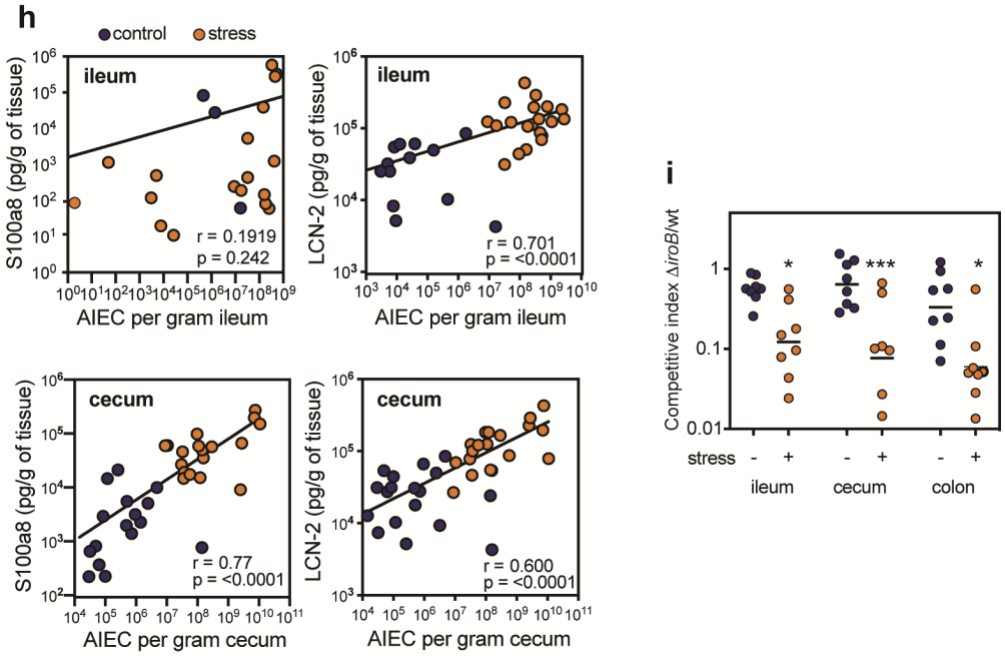
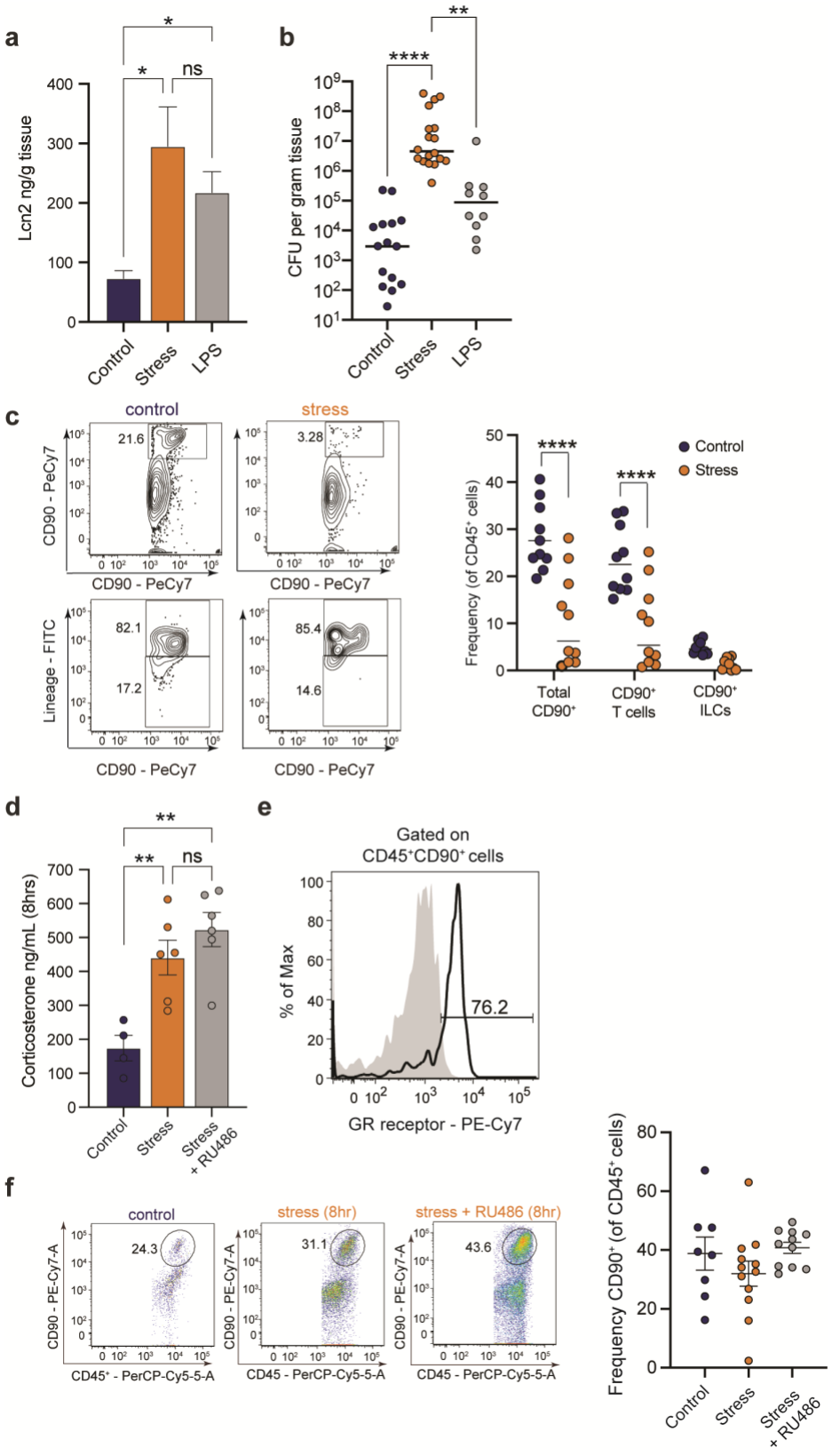


Figure 9. Stress-induces nutritional immunity within the gut and provides a competitive advantage to AIEC. a, Schematic representation of infection and stress protocol. **b,** RT-qPCR analysis from ileal samples of AIEC-colonized starved (n = 12 - 16) or stressed (n = 12 - 16) mice. Significance was determined by two-way ANOVA. **c,** Quantification of Lcn2 by ELISA along the length of the intestinal tract in AIEC-colonized starved (n = 8) and stressed (n = 8) mice. The small intestine was divided into four 8 cm segments in which segment 4 was proximal to the cecum. Significance was determined by two-way ANOVA. **d,** Quantification of the calprotectin subunit S100a8 along the length of the intestinal tract in AIEC-colonized starved (n = 8) and stressed (n = 8) mice. Significance was determined by two-way ANOVA. **e,** RT-qPCR analysis of *S100a9* and *Lcn2* RNA expression in the liver and ileum of AIEC-colonized mice following 4 h of starvation (ileum n = 8, liver n = 4) or stress (ileum n = 8, liver = 4).

Significance to control was determined by one-way ANOVA. **f,** RT-qPCR analysis of Lcn2 in the ileum. Samples were collected from AIEC-colonized mice at baseline as a control, 4 h and 16 h of overnight stress, and at 6 h and 12 h following restraint release (for all time points n = 8). Significance to control was determined by one-way ANOVA. **g,** AIEC fecal burdens collected from mice at baseline as a control (n = 11), 16 h of stress (n = 12), and at 6 h (n = 12) and 12 h (n = 11) following restraint release and recovery. Significance to control was determined by one-way ANOVA. **h,** Spearman correlation of either calprotectin subunit S100a8 concentration or Lcn2 concentration and AIEC fecal burdens in the ileum and cecum (n = 40). **i,** Competitive infection of wild type AIEC and Δ*iroB* in starved (n = 8) and stressed (n = 8) mice. Mice were infected with a 1:1 ratio of wildtype:Δ*iroB* and were subjected to overnight starvation or stress two days later. Following stress or starvation, ileum, cecum, and colon tissues were collected and the ratio of wild type:Δ*iroB* determined by selective plating. Significance was determined by a two-way ANOVA. (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). Error bars represent SEM and the line in CFU graphs indicates the geometric mean of the group.


a
Lcn2 ng/g tissue
Control
Stress
LPS
ns
b
CFU per gram tissue
c
control
stress
CD90 - PeCy7
Lineage - FITC
21.6
3.28
82.1
17.2
85.4
14.6
Frequency (of CD45+ cells)
Total CD90+
CD90+ T cells
CD90+ ILCs
d
Corticosterone ng/mL (8hrs)
Stress + RU486
e
Gated on CD45+CD90+ cells
% of Max
76.2
GR receptor - PE-Cy7
f
stress (8hr)
stress + RU486 (8hr)
CD90 - PE-Cy7-A
CD45+ - PerCP-Cy5-5-A
CD45 - PerCP-Cy5-5-A
24.3
31.1
43.6
Frequency CD90+ (of CD45+ cells)

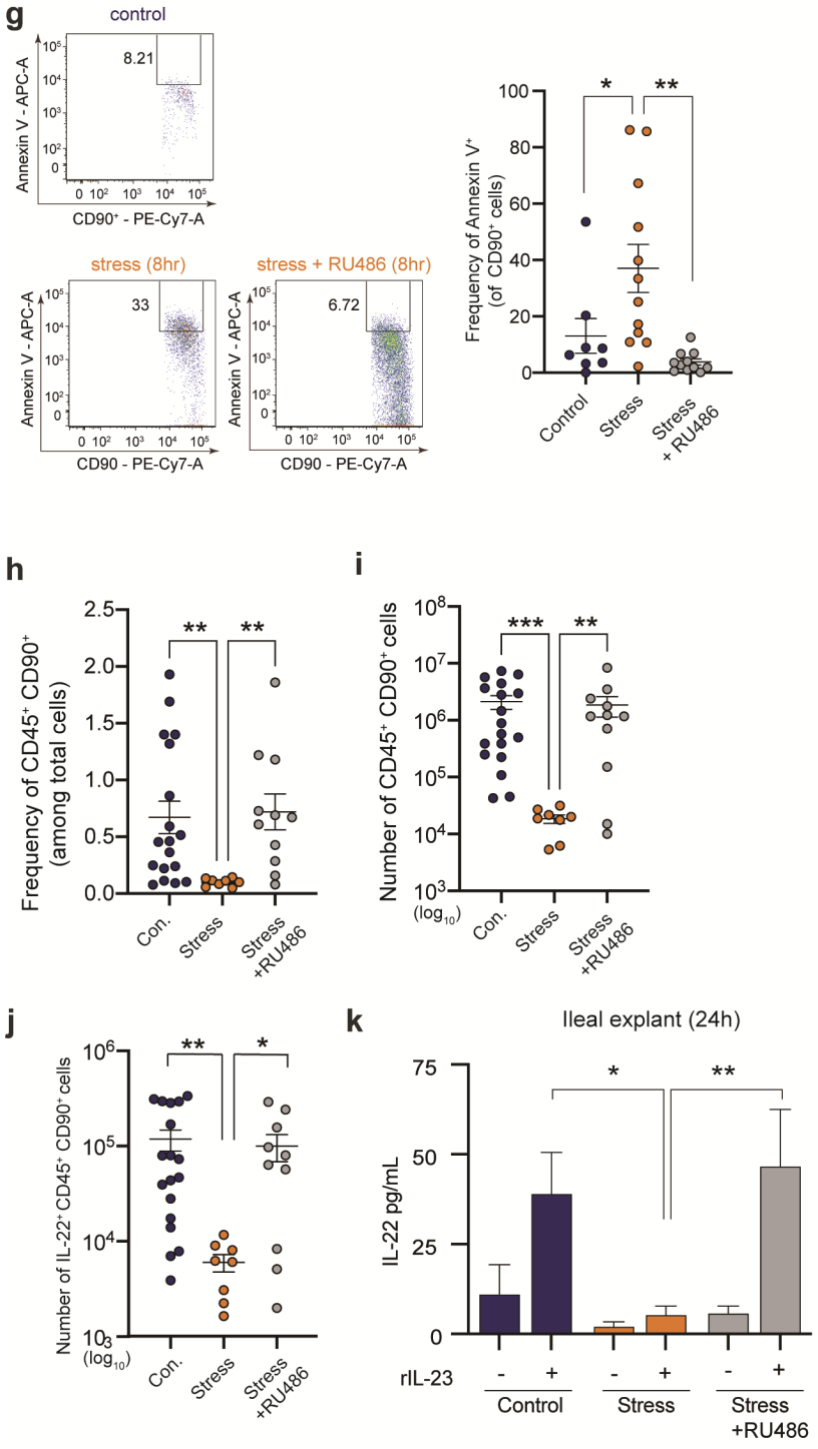

g
control
8.21
Annexin V - APC-A
CD90⁺ - PE-Cy7-A
stress (8hr)
33
stress + RU486 (8hr)
6.72
CD90 - PE-Cy7-A
Frequency of Annexin V⁺ (of CD90⁺ cells)
Control
Stress
Stress + RU486
h
Frequency of CD45⁺ CD90⁺ (among total cells)
Con.
Stress
Stress +RU486
i
Number of CD45⁺ CD90⁺ cells
(log10)
j
Number of IL-22⁺ CD45⁺ CD90⁺ cells
k
Ileal explant (24h)
IL-22 pg/mL
rIL-23
Control
Stress
Stress +RU486

Figure 10: Psychological stress induces attrition of CD90$^+$ cells, resulting in defects in the IL-22 pathway. a, Quantification of ileal Lcn2 as determined by ELISA in control (n = 16), stress (n = 16), and LPS treated (n = 8) mice. Significance was determined by a one-way ANOVA. **b,** Ileal AIEC tissue burdens from control (n = 16), stress (n = 18), and LPS treated (n = 10) mice. **c,** Representative flow plots of CD45$^+$CD90$^{+/-}$ cells and lineage staining on CD45$^+$CD90$^+$. Percentage of CD90$^+$ T cells and CD90$^+$ lineage$^-$ ILCs of total CD45$^+$ cells isolated from ileal lamina propria cells collected from starved (n = 10) and stressed (n = 10) mice as determined by flow cytometry. Significance was determined by two-way ANOVA. **d,** Quantification of corticosterone in the serum as determined by ELISA in control (n = 4), stress (n = 6), and RU486 treated stress (n = 6) mice. Significance was determined by one-way ANOVA. **e,** Representative flow plot of percentage of CD45$^+$CD90$^+$GR$^+$ cells. **f,** Frequency of CD45$^+$CD90$^+$ cells following 8 h of restraint in control (n = 8), stress (n = 12), and RU486 treated stress (n = 11) mice. **g,** Frequency of CD45$^+$CD90$^+$AnnexinV$^+$ cells following 8 h of restraint in control (n = 8), stress (n = 12), and RU486 treated stress (n =11). Significance was determined by a one-way ANOVA. **h,** Frequency of CD45$^+$CD90$^+$ cells following overnight restraint in control (n = 18), stress (n = 8), and RU486 treated stress (n = 11). Significance was determined by one-way ANOVA. **i,** Absolute number of CD45$^+$CD90$^+$ cells following overnight restraint in control (n = 18), stress (n = 8), and RU486 treated stress (n = 11) mice. **j,** Percentage of IL-22$^+$ cells from the absolute number of CD45$^+$CD90$^+$ cells isolated from ileal lamina propria cells collected from starved (n = 14), stressed (n = 8), and RU486 treated (n =10) mice as determined by flow cytometry. Significance was determined by

one-way ANOVA. k, Quantification of IL-22 in the supernatants of ileal explants from starved (n = 10), stressed (n = 10), RU486 treated stress (n = 16) mice stimulated with 20 ng/mL rIL-23 or media control for 24 h. Significance was determined by two-way ANOVA. (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$. Error bars represent SEM and the line in CFU graphs indicates the geometric mean of the group.

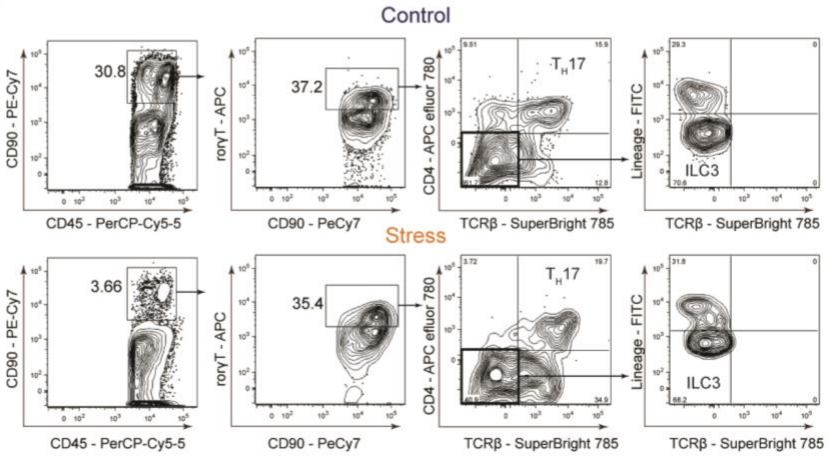
a

Of CD45⁺	Control	Stress
CD90⁺ TCRβ⁺	58.76% ± 6.7%	58.46% ± 10%
CD90⁺ TCRβ-	41.24% ± 3.4%	41.54% ± 7.3%

b

Of TCRβ⁺CD90⁺	Control	Stress
CD4⁺	32.59% ± 5.0%	23.97% ± 4.8%
CD4⁺CD8⁺	20.24% ± 4.4%	20.59% ± 5.9%
CD4⁺CD8⁻	36.28% ± 3.9%	39.28% ± 5.0%
CD4⁻CD8⁻	10.89% ± 1.2%	16.16% ± 2.7%

c



Of CD90⁺	Control	Stress
T_H17	7.7 ± 1.7%	5.4 ± 0.5%
ILC3	5.7 ± 3.5%	4.4 ± 1.2%

Total	Control	Stress
T_H17	$3x10^5 \pm 2x10^5$	$4x10^4 \pm 2x10^4$
ILC3	$7x10^5 \pm 6x10^5$	$2x10^4 \pm 9x10^3$

Figure 11: Psychological stress leads to an unbiased loss of the CD90+ immune population. **a,** Proportion of $CD90^{+}TCRb^{+}$ and CD $CD90^{+}TCRb^{-}$ in control (n = 10) and stress (n = 5) mice. **b,** Proportion of $CD90^{+}TCRb^{+}$ that are $CD4^{+}$, $CD8^{+}$, $CD4^{+}CD8^{+}$, or $CD4^{-}CD8^{-}$ $CD90^{+}TCRb^{+}$ in control (n = 10) and stress (n = 5). **c,** Gating strategy for T_H17 and ILC3 from $CD45^{+}CD90^{+}$.

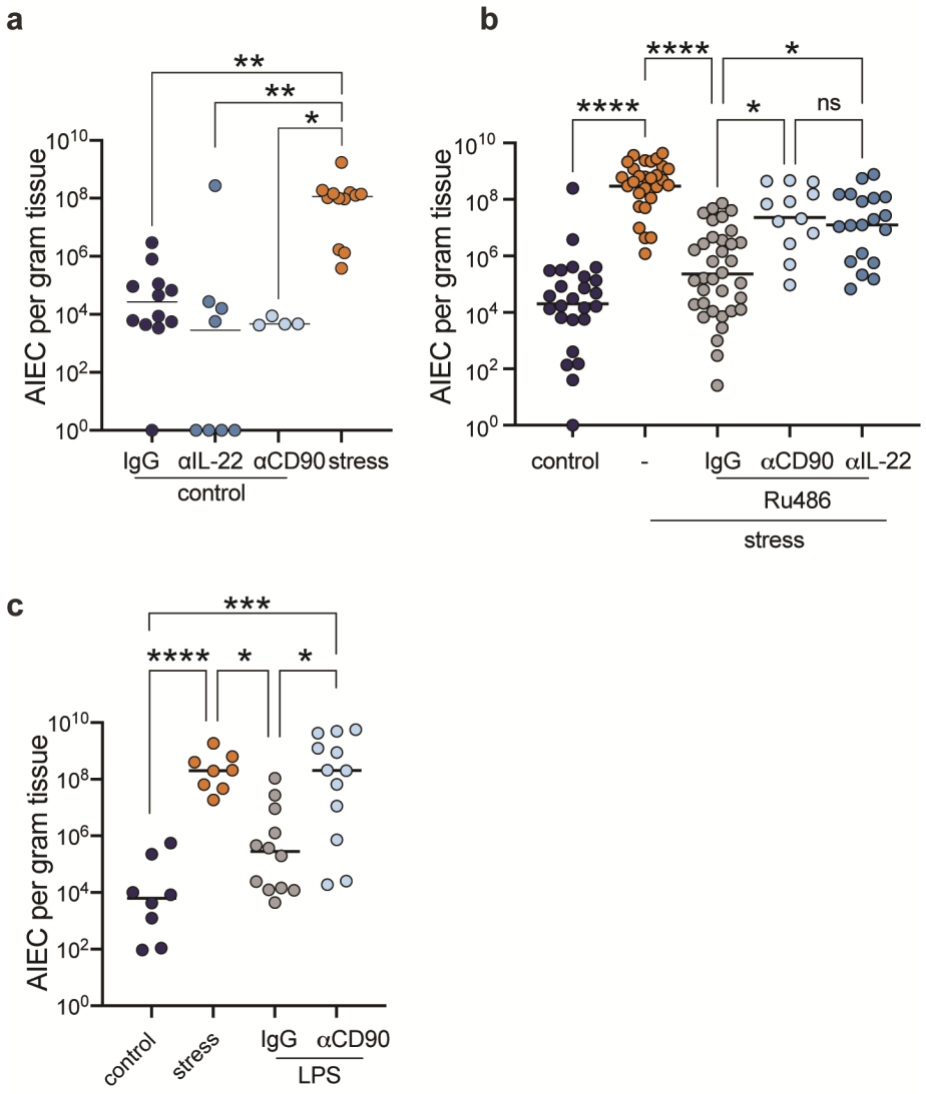
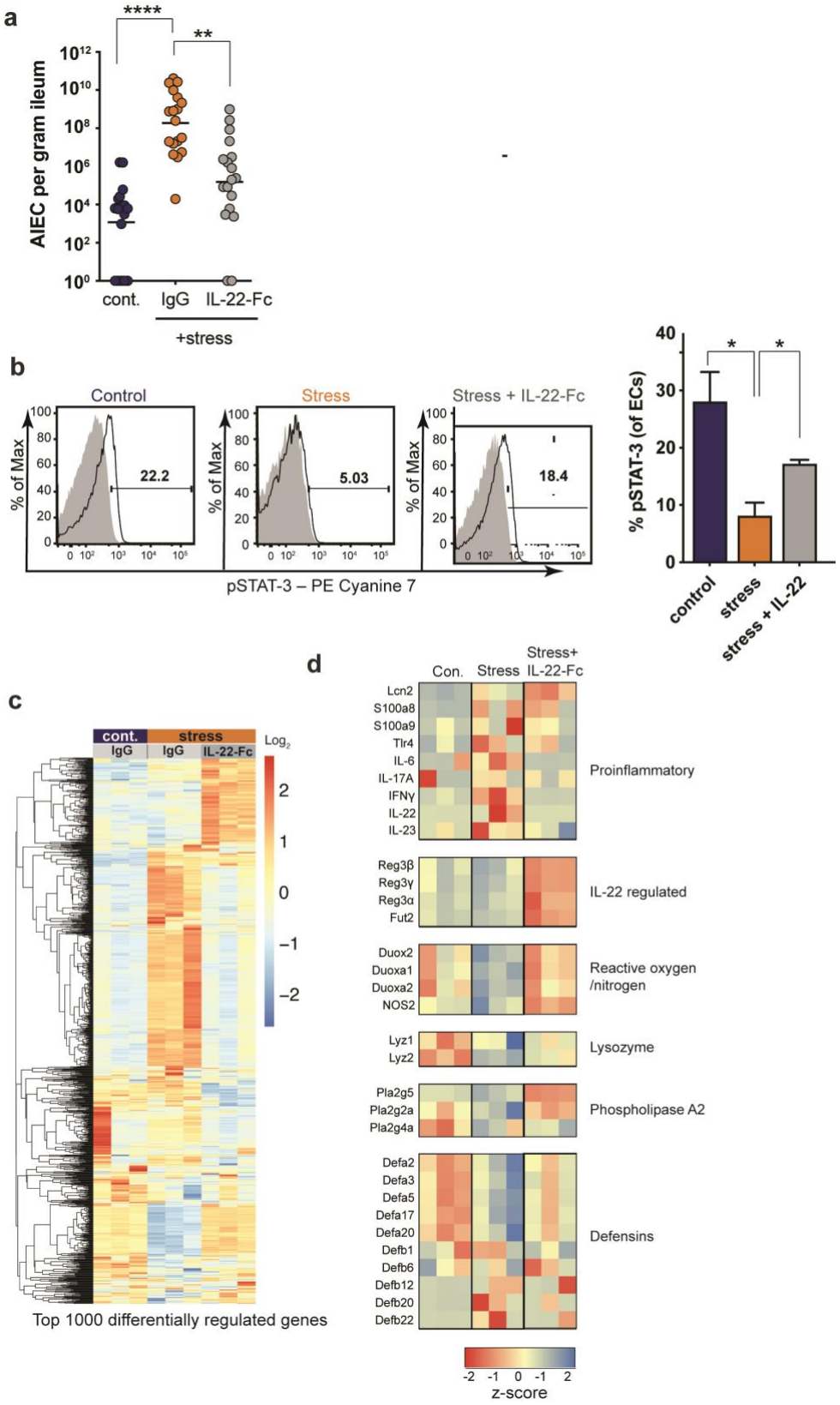

a
**
**
*
AIEC per gram tissue
10^10
10^8
10^6
10^4
10^2
10^0
IgG
αIL-22
αCD90
stress
control
b
****
*
****
*
ns
AIEC per gram tissue
control
-
IgG
αCD90
αIL-22
Ru486
stress
c
***
****
*
*
AIEC per gram tissue
control
stress
IgG
αCD90
LPS

Figure 12: Combinatorial effects of nutritional immunity and immune depletion are responsible for intestinal dysbiosis. a, Ileal AIEC tissue burdens from control (n = 12), aIL-22 (n = 8), aCD90 treated (n = 4), and stress (n = 12) mice. Significance was determined by one-way ANOVA. **b,** Ileal AIEC tissue burdens from control (n = 19), stress (n = 22), RU486 treated stress (n = 25), RU486 and aCD90 treated stress (n = 12), and RU486 and aIL-22 treated stress (n = 13). Significance was determined by one-way ANOVA. **c,** Ileal AIEC tissue burden in control (n = 24), stress (n = 18), LPS treated (n = 22), and LPS and aCD90 treated (n = 12) mice. Significance was determined by one-way ANOVA. (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$. Error bars represent SEM and the line in CFU graphs indicates the geometric mean of the group.


a
AIEC per gram ileum
cont.
IgG
IL-22-Fc
+stress
b
Control
Stress
Stress + IL-22-Fc
% of Max
22.2
5.03
18.4
pSTAT-3 – PE Cyanine 7
% pSTAT-3 (of ECs)
control
stress
stress + IL-22
c
cont.
stress
IgG
IgG
IL-22-Fc
Log2
Top 1000 differentially regulated genes
d
Con.
Stress
Stress+ IL-22-Fc
Lcn2
S100a8
S100a9
Tlr4
IL-6
IL-17A
IFNγ
IL-22
IL-23
Proinflammatory
Reg3β
Reg3γ
Reg3α
Fut2
IL-22 regulated
Duox2
Duoxa1
Duoxa2
NOS2
Reactive oxygen /nitrogen
Lyz1
Lyz2
Lysozyme
Pla2g5
Pla2g2a
Pla2g4a
Phospholipase A2
Defa2
Defa3
Defa5
Defa17
Defa20
Defb1
Defb6
Defb12
Defb20
Defb22
Defensins
z-score

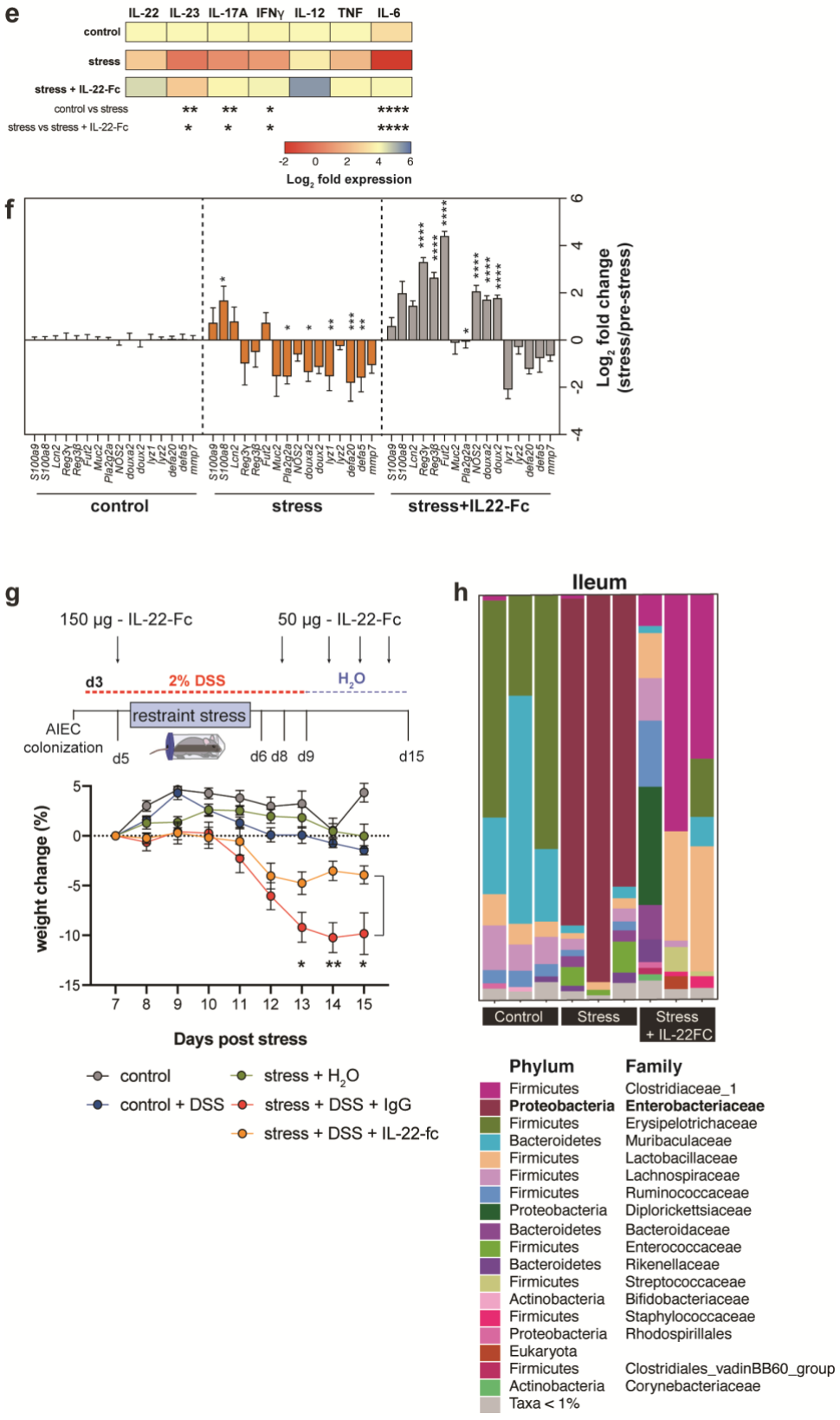

e
IL-22
IL-23
IL-17A
IFNγ
IL-12
TNF
IL-6
control
stress
stress + IL-22-Fc
control vs stress
stress vs stress + IL-22-Fc
Log2 fold expression
f
Log2 fold change (stress/pre-stress)
control
stress
stress+IL22-Fc
g
150 µg - IL-22-Fc
50 µg - IL-22-Fc
d3
2% DSS
H2O
restraint stress
AIEC colonization
d5
d6 d8
d9
d15
weight change (%)
Days post stress
control
control + DSS
stress + H2O
stress + DSS + IgG
stress + DSS + IL-22-fc
h
Ileum
Control
Stress
Stress + IL-22FC
Phylum
Family
Firmicutes Clostridiaceae_1
Proteobacteria Enterobacteriaceae
Firmicutes Erysipelotrichaceae
Bacteroidetes Muribaculaceae
Firmicutes Lactobacillaceae
Firmicutes Lachnospiraceae
Firmicutes Ruminococcaceae
Proteobacteria Diplorickettsiaceae
Bacteroidetes Bacteroidaceae
Firmicutes Enterococcaceae
Bacteroidetes Rikenellaceae
Firmicutes Streptococcaceae
Actinobacteria Bifidobacteriaceae
Firmicutes Staphylococcaceae
Proteobacteria Rhodospirillales
Eukaryota
Firmicutes Clostridiales_vadinBB60_group
Actinobacteria Corynebacteriaceae
Taxa < 1%

Figure 13. IL-22 delivery corrects stress-induced intestinal dysbiosis. a, AIEC tissue burdens collected from the ileum of starved (n = 18), IgG treated stress (n = 18), and IL-22 treated stress (n = 18) mice. Significance was determined by two-way ANOVA. **b,** Representative FACS plots of phospho-STAT-3^{+} cells from isolated epithelial cells. Percentage of phospho-STAT-3 on ileal epithelial cells of starved (n = 4), IgG treated stress (n = 4), and IL-22 treated stress (n = 4) mice as determined by flow cytometry. Significance was determined by two-way ANOVA **c,** RNA sequencing analysis of the top 1,000 differentially regulated genes from ileal samples of AIEC-colonized starved (n = 3), stressed (n = 3), and IL-22 treated, stressed (n = 3) mice. **d,** Z-score values from RNA sequencing analysis of innate immune pathways from ileal samples of AIEC-colonized starved (n = 3), stress (n = 3), and IL-22 treated stress (n = 3) mice. **e,** RT-qPCR analysis of cytokine expression from ileal samples of AIEC-colonized starved (n = 8), IgG treated stress (n = 8), and IL-22 treated stress (n = 8) mice. Control and stress samples are the same as Figure 9b. Significance was determined by two-way ANOVA. **f,** RT-qPCR analysis of AMP expression from ileal samples of AIEC-colonized starved (n = 8), IgG treated stress (n = 8), and IL-22 treated stress (n = 8) mice. Significance was determined by two-way ANOVA. Stress significance is relative to control. Stress + IL-22 significance is relative to stress. Control and stress samples are the same as Figure 8b. **g,** Schematic representation of DSS treatment schedule. The graph depicts weight change normalized two days post stress, (control n = 4, control + DSS n = 7, stress + H_2O n = 4, stress + DSS n = 7, stress + DSS + IL-22-Fc n = 6). Significance between stress + DSS and stress + DSS + IL-22-Fc was determined by Multiple t-tests. **h,** Taxonomy plots of

16s rRNA sequencing of the ileal contents of starved (n = 3), IgG treated stress (n = 3), and IL-22 treated stress (n = 3) mice. (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$). Error bars represent SEM and the line in CFU graphs indicates the geometric mean of the group.

Table 1: Primer sequences for RT-qPCR

Primer Name	Sequence (5' to 3')
IL-5 F	GCAATGAGACGATGAGGCTTC
IL-5 R	GCCCCTGAAAGATTTCTCCAATG
IL-6 F	TAGTCCTTCCTACCCCAATTTCC
IL-6 R	TTGGTCCTTAGCCACTCCTTC
IL-10 F	CTTACTGACTGGCATGAGGATCA
IL-10 R	GCAGCTCTAGGAGCATGTGG
IL-12 F	GTCCTCAGAAGCTAACCATCTCC
IL-12 R	CCAGAGCCTATGACTCCATGTC
IL-13 F	TGAGCAACATCACACAAGACC
IL-13 R	GGCCTTGCGGTTACAGAGG
IL-17A F	TGAGAGCTGCCCCTTCACTT
IL-17A R	ACGCAGGTGCAGCCCA
IL-22 F	GGCCAGCCTTGCAGATAACA
IL-22 R	GCTGATGTGACAGGAGCTGA
IL-22BP F	AAGCATTGCCTTCTAGGTCTCC
IL-22BP R	TCAGAGATACACGAGCTGGTT
IL-23 F	TTCAGATGGGCATGAATGTTTCT
IL-23 R	CCAAATCCGAGCTGTTGTTCTAT
IL-33 F	CAATCAGGCGACGGTGTGGATGG
IL-33 R	TCCGGAGGCGAGACGTCACC

IFN-γ F	TCAAGTGGCATAGATGTGGAAGAA
IFN-γ R	TGGCTCTGCAGGATTTTCATG
TNF F	ACGGCATGGATCTCAAAGAC
TNF R	AGATAGCAAATCGGCTGACG
TLR4 F	ATGGCATGGCTTACACCACC
TLR4 R	GAGGCCAATTTTGTCTCCACA
HMOX-1 F	ACGCATATACCCGCTACCTG
HMOX-1 R	CCAGAGTGTTCATTCGAGCA
HPX F	AGCAGTGGCGCTAAATATCCT
HPX R	CCATTTTCAACTTCGGCAACTC
Haptoglobin F	GCTATGTGGAGCACTTGGTTC
Haptoglobin R	CACCCATTGCTTCTCGTCGTT
mSAA F	AGTGGCAAAGACCCCAATTA
mSAA R	GGCAGTCCAGGAGGTCTGTA
S100a8 F	TGTCCTCAGTTTGTGCAGAATATAAA
S100a8 R	TCACCATCGCAAGGAACTCC
S100a9 F	GGTGGAAGCACAGTTGGCA
S100a9 R	GTGTCCAGGTCCTCCATGATG
Lcn2 F	ACATTTGTTCCAAGCTCCAGGGC
Lcn2 R	CATGGCGAACTGGTTGTAGTCCG
Reg3γ F	TGCTGCTCTCCTGCCTGATG

Reg3γ R	ATAGGAGCCATAGGCACGGG
Reg3β F	ACTCCCTGAAGAATATACCCTCC
Reg3β R	CGCTATTGAGCACAGATACGAG
Fut2 F	ACAGCCAGAAGAGCCATGGC
Fut2 R	TAACACCGGGAGACTGATCC
Muc2 F	ACCTGGGGTGACTTCCACT
Muc2 R	CCTTGGTGTAGGCATCGTTC
GATA4 F	CCCTACCCAGCCTACATGG
GATA4 R	ACATATCGAGATTGGGGTGTCT
Muc1 F	TACCCTACCTACCACACTCACG
Muc1 R	CTGCTACTGCCATTACCTGC
Muc2 F	ACCTGGGGTGACTTCCACT
Muc2 R	CCTTGGTGTAGGCATCGTTC
Muc3 F	CTTCCAGCCTTCCCTAAACC
Muc3 R	TCCACAGATCCATGCAAAAC
Muc4 F	GAGAGTTCCCTGGCTGTGTC
Muc4 R	GGACATGGGTGTCTGTGTTG
Cldn2 F	GGCTGTTAGGCACATCCAT
Cldn2 R	TGGCACCAACATAGGAACTC
Occludin F	TTGAAAGTCCACCTCCTTACAGA
Occludin R	CCGGATAAAAAGAGTACGCTGG

Table 2: Primer sequences for mutant construction

Primer Name	Sequence
NRGiroBKO F	5'ATCTGGTAAAGCAGGTTTAACGAAAAGTCGTGTCCTGCTGCTGGCATATGAGTGCGTCGACTGCCTGATTTAGATCGTCAAGCGGAGAGGGATTTTCTCAAATTCCCCTGTAGAAATAAT 3'
NRGiroBKO R	5'ATCGTTCCGCAAAAAAGCCAGCACGCTCCGGTCCCGCATTGTTATGGATGCATGACTCCTGGATGGGTGCTGGCATATGATTATCATGAAGGTGTAGTGGGGTGATGTGCCATGGGAAGT 3'
iroBScrn F	5' GTGAAGCACAAATGGCGCAG 3'
iroBScrn R	5' AAATACGATCCACTGGCCGG 3'

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