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Commensal Microbiota-Derived Indoles as Determinants of Inflammaging and Healthspan

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ABSTRACT

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By Domonica Nichole Powell

Aging is accompanied by an increase in baseline inflammation throughout the body and a decline in anti-inflammatory processes, a phenomenon known as inflammaging. Additionally, overall dysfunction of the immune system, known as immunosenescence, is observed with age. Inflammaging is associated with an increased risk of becoming frail with age and increased susceptibility to inflammatory age-related diseases. A proposed cause of inflammaging is a decline in intestinal barrier function with age, which allows continuous translocation of luminal antigens that can initiate a chronic inflammatory immune response. Moreover, dysbiosis associated with age-related diseases indicates a connection between the intestinal microbiota and inflammaging.

The intestinal epithelium is a highly dynamic structure that is continuously regenerating through intestinal stem cell proliferation and differentiation into absorptive and secretory cell types. Intestinal goblet cells are responsible for secreting mucus that protects the epithelium by limiting access of pathogens and lubricating the intestinal tract to prevent abrasion. Together, the mucus layers and intestinal epithelium form a highly integrated barrier system that limits contact of the intestinal microbiota with the host while still permitting access of small molecules produced by commensal bacteria.

This work demonstrates that geriatric mice lose goblet cells as they age, and small molecules related to indole, either provided through colonization with bacteria that secrete indole and its derivatives or administration of the indole derivative indole-3 aldehyde (ICA), can restore goblet cell numbers to those seen in young mice. Increased goblet cell differentiation in response to indoles was dependent on an aryl hydrocarbon receptor (Ahr)-mediated increase in expression of the immunoregulatory cytokine interleukin-10 (IL10). Thus, indoles derived from the commensal microbiota regulate intestinal homeostasis, especially during aging, when intestinal barrier function declines. Additionally, IL10 induced by commensal microbiota-derived indoles may function in organ systems outside of the intestine, such as skeletal muscle, to reduce the negative consequences of inflammaging.

Together, this work demonstrates that indoles may have utility as a therapeutic intervention to both limit the decline of barrier integrity by restoring goblet cell numbers and control systemic inflammation via the anti-inflammatory cytokine IL-10.

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CHAPTER I

Introduction

Overview

The capacity to maintain barrier integrity is essential for survival in diverse organisms, ranging from *Caenorhabditis elegans* and *Drosophila melanogaster* to mammals. External surfaces of multicellular organisms such as the skin, the female reproductive tract, and the digestive system act to separate essential internal organs from the outside world and are the body's first line of defense against infection and environmental damage. The outside of these barrier surfaces is extensively colonized with microorganisms, which are collectively referred to as the microbiota. Studies using germ-free model organisms without a microbiota have demonstrated that colonization with bacteria is crucial for normal development and physiology (1, 2). Therefore, in addition to providing protection, barriers must also allow for interaction between the host and the microbiota (3). Accordingly, organisms have evolved delicately regulated barrier systems that permit communication with the environment while still excluding microorganisms from the sterile inner compartment of the body (4).

The significance of the intestinal barrier system is made evident by the consequences of barrier breakdown. Damage to or dysregulation of the barrier allows bacteria to translocate into the sterile inner compartment of the body, leading to infections, inflammatory responses, and potentially systemic disease (5). Therefore, to maintain homeostasis, the barrier must be a dynamic system capable of rapid repair and regeneration in response to damage. Additionally, immunoregulatory processes are critical for preventing or containing inflammatory responses (6). Identification of factors that regulate these processes are of great utility to both prevent and treat

maladies linked to barrier dysfunction. A recent boom in microbiota research has generated intense interest in identifying specific microbes or microbial products that improve the health of the intestinal barrier to protect against infection and environmental damage. The work set forth in this dissertation investigates how a family of small molecules known as indoles, either derived from the commensal microbiota or provided via supplementation, influence intestinal homeostasis through modulation of the intestinal barrier and immunoregulatory processes. Critically, the capacity of indoles to restore dysregulated homeostatic processes during aging illuminates a potential therapeutic for the treatment of frailty and age related diseases.

The Barrier System of the Colon

The barrier system of the colon comprises both an epithelial barrier and overlying mucus layers. The mucus limits the contents of the lumen, including the microbiota, potential pathogens, and food waste, to the epithelium while still permitting access of small molecules such as microbial metabolites. The epithelial barrier further limits access of lumenal contents to the body. This system provides a vital defensive barrier separating the body from the microbial populations residing in the intestinal lumen. Indeed, growing evidence shows that loss of either component of this barrier system may cause disease, both locally and systemically, as the result of bacteria or antigens entering the body (7, 8). A comprehensive understanding of the epithelial and mucus barriers and their relationship to commensal microbes and to the immune system may provide a more integrated approach to treatment of conditions associated with barrier dysfunction.

Epithelial Barrier

The colonic epithelium is a monolayer of billions of contiguous cells organized into crypt structures that increase surface area for water and nutrient absorption. When at homeostasis, this epithelial barrier is highly dynamic and undergoes a continuous rapid turnover process that is crucial for removal and replacement of damaged or infected cells. $Lgr5^+$ crypt base columnar (CBC) stem cells located at the base of crypts of Lieberkühn divide daily to regenerate this monolayer during homeostasis (9). $Bmi1^+$ reserve stem cells located at the +4 position from the base of the crypt are normally quiescent and are activated in response to damage, for example after radiation exposure (10). The daughter cells resulting from stem cell divisions continue to divide as they move up through the middle of the crypt, a region known as the transit amplifying zone, and begin to terminally differentiate into specialized cells as they migrate. Differentiation proceeds along either an absorptive lineage, comprising colonocytes that absorb water and maintain electrolyte balance, or a secretory lineage, comprising mucin secreting goblet cells, hormone producing enteroendocrine cells, and rare Tuft cells, depending on differentiation factors to which the cells are exposed (9). The small intestine additionally contains antimicrobial secreting Paneth cells, but this cell type is absent in the colon, where there instead is an abundance of goblet cells (11). Cells that reach the crypt surface eventually undergo anoikis, a form of programmed cell death that occurs when cells detach from the epithelium, and are shed into the lumen. Peristalsis, smooth muscle contractions of the intestine, then facilitates the removal of waste from the body. This regenerative cycle from stem cell division at the base of the crypt to anoikis at the lumen is complete within a few days (11).

Maintaining continuous proliferation of intestinal stem cells (ISCs) is critical for the epithelial regenerative cycle and depends on Wnt and Notch signaling pathways working

cooperatively (12, 13). Wnt ligands are produced by stromal cells in the crypt base niche and bind to the transmembrane receptor Frizzled (FZD) along with its coreceptor low-density lipoprotein receptor-related protein (LRP)(14). The resulting signaling cascade culminates with ß-catenin entering the nucleus and forming complexes with T-cell factor (TCF) to transcribe genes that regulate the cell cycle (15). Inducible deletion of ß-catenin in mice blocks stem cell proliferation resulting in a loss of crypt architecture and intestinal function (16) Correspondingly, loss of the Wnt negative regulator Adenomatous Polyposis Coli (APC) results in increased stem cell proliferation and initiates the development of colorectal polyps and cancer (17, 18). These studies demonstrate that Wnt signaling is essential for stem cell proliferation. Notch signaling is also required for stem cell proliferation, although it is dependent on Wnt signaling in those cells; Notch signaling alone is not sufficient (19). Overexpression of Notch results in expansion of proliferating cells at the expense of differentiation, and Notch inhibition or deletion of Notch ligands results in the loss of stem cells and conversion of proliferating cells to differentiated cell types (20, 21). Notch signaling is also thought to regulate the balance of active $Lgr5^+$ stem cells and quiescent $Bmi1^+$ stem cells (10).

Differentiation of daughter cells must be controlled in order to maintain an appropriate balance between absorptive and secretory cells and thus preserve a functional barrier. Regulation of differentiation is largely dictated by Notch lateral inhibition signaling. The transmembrane Notch receptor (mainly Notch1 in the colon) is activated by membrane bound ligands (Delta-like ligand (DLL) 1-4 and Jagged 1-2) on adjacent cells, which results in a series of proteolytic cleavages, including one by gamma-secretase, that release the active Notch Intracellular Cleaved Domain (NICD). NICD translocates from the cytoplasm to the nucleus where it heterodimerizes with CSL to transcribe target genes, including *Hes1*. Hes1 inhibits Atoh1/Hath1/Math1, a factor

required for secretory cell differentiation. Thus, Notch signaling results in differentiation to absorptive epithelial cells. In the presence of Notch inhibitors, such as gamma-secretase inhibitor dibenzazepine (DBZ), or with deletion of the Notch effector *Hes1*, *Atoh1* expression increases, and differentiation is preferentially along the secretory lineage, resulting in goblet cell hyperplasia (21–24). Constitutively active NICD results in a loss of secretory cells (20). *Atoh1* deletion concomitantly with Notch blockade prevents goblet cell hyperplasia, indicating that Atoh1 is necessary for secretory cell differentiation (25). Therefore, Notch, Hes1, and Atoh1 are important factors in determining the fates of differentiating cells in intestinal crypts.

Wnt also seems to play a role in making cells competent to become secretory cells. Overexpression of the Wnt inhibitor Dickkopf1 (Dkk1) results in the absence of secretory cells, while the absorptive cell lineage is unaffected (26). The diffusible Wnt gradient is highest at the base of the crypt and prevents cells from fully differentiating until they exit the transit amplifying zone. $Wnt^{+}Notch^{+}$ cells become absorptive precursors that fully differentiate into absorptive enterocytes. $Wnt^{+}Notch^{-}$ cells become secretory precursor cells and eventually one of the four types of differentiated secretory cells depending on which additional factors are present (13). Additional expression of the transcription factors Klf4 and SPDEF are required for terminal differentiation and full maturation of goblet cells from a secretory progenitor (27, 28).

All epithelial cells of the intestine are attached to adjacent cells by adhesive contacts called junctions, which form a paracellular seal around the cell. The seal between cells requires specialized adhesion structures called tight junctions (TJs). The TJs are positioned at the boundary of the apical and lateral membrane surfaces of adjacent epithelial cells and consist of 5–7 membrane fusion sites called kissing points (29, 30). The entire circumference of each cell is joined to adjacent cells via an adhesive TJ band, called a strand. TJs perform multiple functions including

regulating cell polarity by corralling apical membrane proteins and anchored mucins to the lumenal surface of the epithelium, called the fence function, and regulating solute and ion flux through the paracellular space, called the gate function, which is especially important in forming a barrier against bacteria and bacterial products (31, 32).

The claudin family proteins are essential components of TJs. Claudins form TJ strands by polymerizing within the plasma membrane and dimerizing with claudins on adjacent cells, across the extracellular space, to generate the paracellular seal (33). Humans possess 24 different claudin genes, and different claudins can form either ion pores (ex. claudin 2 and 15) or tight seals (ex. claudin 4 and 7)(34, 35). The balance of claudins forming pores or seals contribute to the overall permeability of the TJ (32, 36). Sealing claudins are predominantly expressed at the lumenal surface where more strict barrier function is required in the face of microbial and food antigens, while pore forming claudins are primarily expressed at the base of crypts where antigen load is typically lower (35, 37). In addition to forming pores, claudin strands have a poorly understood mechanism that permits small molecules to traverse the TJ, termed the paracellular leak pathway (38, 39). There is accumulating evidence that TJ strands themselves are dynamic and frequently break, reform, and exchange claudin proteins in response to physiological environmental, and pathogenic stimuli (35). In summary, TJ dynamics and claudin composition and localization within the intestine all contribute to the permeability of the intestinal barrier.

Mucus Barrier

Rather than coming in direct contact with the intestinal epithelium, the intestinal microbiota is sequestered by mucus (40, 41). The mucus barrier, composed of mucin proteins secreted by goblet cells, functions as a means of limiting contact of bacteria and bacterial antigens with the

epithelial barrier and additionally provides protection against the mechanical stress of food waste passing through the intestine (42). The unique two-layer structure of colonic mucus allows for all of these functions concurrently; a bacteria-impenetrable inner layer provides protection against the microbiota and potential pathogens, and a looser outer layer houses the microbiota and helps lubricate the passage of fecal material (43).

The structure of the mucus barrier in the colon is distinct from that of the small intestine (44). Since the concentration of bacteria in the lumen increases from the small intestine to the colon, more stringent control measures are likely required in the colon to segregate the microbiota (45, 46). The small intestine contains a single layer of loose unattached mucus that is penetrable by bacteria, and antimicrobial peptides produced by Paneth cells are mainly responsible for keeping bacteria distanced from the epithelium (43, 47). In contrast, mucus in the colon is organized into two layers. The mucin glycoprotein Muc2 forms a tightly crosslinked inner mucus layer that excludes bacteria while still allowing microbiota or dietary derived proteins and small molecules to pass through pores and reach the epithelial surface. This inner mucus layer is firmly attached to the epithelium via membrane-anchored mucins, including Muc1 and Muc3, that form the glycocalyx of colonic epithelial cells. Studies in *Muc2* deficient mice reveal that without the inner mucus layer, bacteria directly contact the colonic epithelium and enter crypts, leading to inflammation, spontaneous colitis, and potentially colon cancer, illuminating the essential role mucus plays in isolating the host from the microbiota (41, 48). The outer mucus layer is formed by proteolysis of the inner layer, generating loose mucus that is highly accessible to bacteria that bind carbohydrate motifs, immunoglobulins, and other proteins. The proteolysis is thought to occur through combination of host and bacteria derived enzymes. Host enzymes are sufficient for degrading the inner layer as germ-free mice still have an outer mucus layer, although they do have

a thicker outer layer without bacteria (41). The mucus layers turn over in a matter of hours, providing continual removal of bacteria and limiting their access to the epithelium (49).

Goblet cells continuously produce copious amounts of Muc2. Following translation, Muc2 proteins form dimers at their C terminals in the endoplasmic reticulum and are then extensively O-glycosylated along their PTS (proline, threonine, serine) domains in the Golgi apparatus (50). Muc2 glycoproteins then form large disulfide-linked trimers through their N termini and are stored in secretory granulae at the apical surface at the goblet cell. Upon secretion from goblet cell granulae into the lumen, Muc2 macromolecules become hydrated and expand 100-1,000-fold in volume to form a net-like gel, forming the tightly crosslinked and bacteria-impenetrable inner mucus layer (42). Secretion of Muc2 by goblet cells is spatially regulated within the colonic crypts. Goblet cells at the lumenal surface of the epithelium continuously secrete Muc2 without a stimulus in order to replenish the mucus. Goblet cells in other locations require stimuli to induce mucus secretion. Goblet cells towards the base of colonic crypts are stimulated by acetylcholine or histamine to secrete Muc2 (51). Goblet cells towards the top of the crypts accumulate more Muc2 within their secretory granulae and can be stimulated by endocytosis of bacterial antigens that penetrate the inner mucus layer. These “sentinel” goblet cells respond to Toll-like receptor (TLR) ligands, including lipopolysaccharide (LPS), which then triggers compound exocytosis of the granulae containing Muc2 and expulsion of the sentinel cell from the epithelium. This process can additionally trigger Muc2 secretion from other adjacent goblet cells (52). This mechanism serves to further prevent bacteria from contacting the epithelium and entering the crypts.

In addition to facilitating removal of bacteria, the mucus also provides a habitat for the microbiota by providing structures for adherence. With the constant turnover of mucus, bacteria need to form attachments within the mucus to minimize the chance of being completely removed

(53). Immunoglobulin A (IgA), the major immunoglobulin secreted at mucosal surfaces, is an important regulator of microbiota composition through facilitating colonization of bacteria within the mucus (54, 55). Local factors in the intestine, including TGF-β, IL10, and retinoic acid, dictate class switch to IgA production by B cells (56). IgA is transported to the lumenal surface of the epithelium by the polymeric immunoglobulin receptor (pIgR) where proteolytic cleavage releases the external component of pIgR, known as secretory component (SC). SC remains bound to IgA to protect it from degradation by proteases, and glycans on SC are thought to mediate attachment of IgA to glycosylated Muc2 (55, 57).

IgA in the outer mucus layer may regulate the microbiota in multiple ways. Antigen specific IgA directed against potentially harmful bacteria can help protect against bacterial translocation, inflammation, and colitis (58–60). However, IgA also positively regulates the composition and distribution of the microbiota and is a critical regulator of intestinal homeostasis. Around a quarter of intestinal bacteria are bound to IgA, and yet, there typically is not an active immune response against commensals (61, 62). In B cell deficient mice, intestinal bacterial counts remained static, yet changes in microbiota composition were apparent (63). Additionally, in mice that are unable to diversify IgA through somatic hypermutation, there is an altered IgA repertoire which results in striking shifts in microbiota composition and evidence of a loss of barrier integrity and systemic inflammation (64). Likewise, humans with specific IgA deficiency show mild intestinal dysbiosis and systemic inflammation. Bacteria normally bound to IgA in control subjects were more likely to be underrepresented in the absence of IgA, indicating that binding to IgA may actually promote the presence of some bacteria (65). Together, these studies demonstrate that IgA in the outer mucus layer is an important regulator of microbiota composition and intestinal homeostasis.

The mucus is also an energy source for mucolytic bacteria capable of degrading mucin glycans including the commensals *Bacteroides thetaiotaomicron* and *Akkermansia muciniphila* (66). The ability to bind mucus and utilize the mucin glycans increases the ability of the bacterium to penetrate the mucus and thus increases the likelihood that this species will impact host health, for better or worse. In some cases, mucin-degrading bacteria, including *A. muciniphila* and *Bacteroides fragilis*, are associated with positive health outcomes (66, 67). However, the mucin degrader *Ruminococcus torques* is strongly associated with increased symptom severity in IBD patients indicating that potentially harmful bacteria may also be able to access the epithelium via mucus utilization (68). Non-mucin-degrading bacteria, such as *Escherichia coli*, are also capable of living within the colonic outer mucus since this environment provides additional nutrient sources, including phospholipids and proteins from shed epithelial cells (69). In return for the energy sources provided in the mucus, bacteria produce short chain fatty acids (SCFAs), including butyrate, propionate, and acetate, which are used for energy by epithelial cells (42, 66). SCFAs also have anti-inflammatory effects and can impact intestinal cell proliferation and differentiation (70). The outer mucus layer facilitates complex interdependent relationships between the microbiota and the host and is thus an important regulator of intestinal homeostasis.

The colonic mucus functions dually to distance the microbiota while at the same time supporting its existence and is a key player in how an organism is able to host an enormous number of microbes safely. Despite the microbiota being required, it does post a constant threat to the health of the host. Together, the mucus layers and colonic epithelium cooperate to form a highly integrated barrier system that functions to limit bacterial contact with the host while still permitting access of small molecules. Through in vivo and in vitro studies in experimental models, as well as studies of human IBD, an integrated understanding of mucosal barrier function is beginning to

emerge. The capacity of the mucus to prevent abrasion and trap bacteria represents the first line of defense, while the paracellular TJ barrier prevents leakage of bacterial antigens from the lumen into the body. Furthermore, the rapid turnover of both mucus and lumen-facing epithelial cells ensures that bacteria that do interact with these structures are constantly being evicted. A comprehensive understanding of the epithelial barrier system and its relationship to commensal microbes and to the immune system may provide a more integrated approach to treatment of maladies associated with barrier dysfunction.

Interleukin-10 and Intestinal Homeostasis

Homeostasis in the intestine depends on the barrier system, including epithelial tight junctions and mucus, in order to separate the microbiota and prevent inappropriate activation of the immune system (71). Complex interconnected signaling occurs between the epithelial cells, cells of the immune system, and the microbiota to maintain homeostasis in this dynamic system. While the ability of the body to induce an inflammatory response to intestinal antigens is critical for survival in the event of infection, prolonged and uncontrolled inflammation can lead to deleterious effects. A key mechanism of preventing inflammatory damage is though production of anti-inflammatory factors, including interleukin-10 (IL10) from regulatory T cells (Tregs), that maintain the immune system non-responsive to antigens and promote immune tolerance (72). In the event that an inflammatory response does occur, IL10 also plays a role in containing and eventually resolving any inflammation. In this regard, increased serum levels of IL10 are found in inflammatory bowel disease patients as they enter a phase of remission (73).

Studies utilizing knockout mice have demonstrated that IL10 is a critical regulator of intestinal homeostasis through immunoregulation. Mice lacking IL10 ($Il10^{-/-}$) or a functional IL10

receptor (*Il10rb*$^{-/-}$) develop systemic inflammatory reactions and a spontaneous enterocolitis similar to human Crohn's disease (74, 75). Additionally, mice deficient in IL2, which is required for Treg development and expansion, display a loss of intestinal homeostasis (76). However, when these mice are kept under specific pathogen free conditions, the inflammation is limited to the intestine, indicating that IL10 is essential for regulating responses to inflammatory intestinal antigens (6, 74, 77, 78). Many immune cells present in the intestine, including T cells, B cells, macrophages, and dendritic cells, and intestinal epithelial cells are capable of expressing *Il10* (79, 80). Further studies have elucidated that intestinal homeostasis depends on IL10 derived from Tregs signaling through IL10R on myeloid cells (81–83). Treg derived IL10 signaling in myeloid cells promotes a state of non-responsiveness in intestinal macrophages and dendritic cells to bacterial antigen stimulation via MyD88 signaling (81, 82, 84–87). In the absence of this IL10 signaling, myeloid cells produce more of the inflammatory cytokines IL12p40 and TNFα in response to stimulation (88).

The effects of intestinal IL10 extend beyond immune cells to intestinal epithelial cells. Treatment of human epithelial cells with IL10 increases transepithelial resistance, a measure of barrier integrity from tight junctions. Further, mice without epithelial IL10 signaling have more permeable epithelial barriers and perform worse in response to experimental dextran sodium sulfate (DSS) colitis (89, 90). In response to damage, IL10 from macrophages signals through the intestinal epithelium to promote proliferation and wound repair, a critical requirement for the epithelium to maintain homeostasis (91). Additionally, IL-10 plays a role in promoting mucus production by goblet cells by regulating ER stress and can increase goblet cell differentiation when administered directly to the colon (92, 93). Together, these data demonstrate that in addition to maintaining non-responsiveness to antigen load of the intestine, IL10 plays a role in regulating

homeostasis of the epithelial and mucus barriers through IL10R signaling in intestinal epithelial cells.

Commensal members of the microbiota themselves play a role in promoting immune regulation. SCFAs produced by bacterial fermentation can promote IL10 expression by T cells that then protects against the development of DSS colitis (94). Specific members of the microbiota, including cluster IV and XIVa *Clostridia* strains and *B. fragilis* favor the development of Tregs over the inflammatory Th17 T cell subset (83). Additionally, regulation of IL10R by microbial products can promote anti-inflammatory signaling (90, 95). Together, these studies suggest that dysbiosis can break intestinal homeostasis and drive inflammation. In summary, crosstalk between the immune system, epithelial barrier, and intestinal microbiota is a critical driver of anti-inflammatory and tolerogenic signaling mechanisms that are necessary for maintaining intestinal homeostasis.

Consequences of Intestinal Inflammation

A breakdown of intestinal barrier function or in immune regulation leads to a loss of homeostasis, allowing dissemination of bacteria or bacterial antigens and shifting the balance towards inflammation. Continued presence of stressors that elicit an inflammatory response or a reduced anti-inflammatory response leads to protracted activation of the immune system and chronic unresolved inflammation (96). Persistent inflammation can also further damage the barrier, promoting a complex cycle of inflammation, damage, and dysbiosis (97). In this regard, loss or dysregulation of either the mucus or the TJs suffice to cause colitis, an effect that depends upon bacterial or antigen translocation. Inflammatory bowel diseases and inflammaging are just two examples of inflammatory conditions that have been linked to chronic inflammation originating at

the intestinal barrier. The use of mouse models used to recapitulate human diseases has enriched our understanding of how reduced barrier integrity plays a role in the onset of chronic inflammation.

Inflammatory Bowel Disease

Inflammatory bowel diseases (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic or intermittent inflammation of the intestinal mucosa (98). IBD is an idiopathic and multifactorial condition, and recent studies indicate that both genetic and environmental factors contribute to the disease (97, 99). During the etiology of IBD, immune responses are directed towards the microbiota or bacterial antigens, causing inflammatory responses that progressively degrade the intestinal epithelia. This in turn permits more antigen leak and exacerbates inflammation, further compromising barrier integrity (97). Mucosal barrier integrity, therefore, is a key determinant of disease initiation, progression, and severity in IBD patients. Mutations in IL10 and IL10R have been associated with human IBD (100, 101). However, despite the evidence that IL10 is critical in maintaining intestinal homeostasis and preventing spontaneous enterocolitis in mouse models, treatment with recombinant IL10 has not been successful (102). Rather, directly disrupting the inflammatory feedback cycle is a fundamental goal of IBD therapy. In experimental models, exposure of epithelial cells to proinflammatory cytokines such as tumor necrosis factor-α (TNFα) causes cell death, dysregulates tight junction proteins, alters the production of secreted mucins, and disrupts the epithelial barriers (103–106). Notably, the anti-TNFα monoclonal antibody, infliximab, a treatment for advanced IBD, short-circuits this inflammatory feedback loop by limiting immune activation and mitigating cytotoxic effects (105). Studies in mice and humans have identified factors in both epithelial and

mucus barriers and in the microbiota that when dysregulated or mutated contribute to loss of barrier function and onset of disease.

Several lines of evidence implicate dysregulation of the epithelial barrier, and of TJ architecture and claudin expression in particular, in the etiology of IBD (30, 104). GWAS studies have identified several genes that link TJ function to IBD (99, 107). Of the IBD implicated genes, one of the best understood is hepatocyte nuclear factor alpha (HNF4α), a transcription factor involved in the maturation of colonocytes as they migrate out of the crypts and in regulating claudin expression, including claudin 7 (108, 109). HNF4α is mutated in 10% of colitis patients. Moreover, expression of the sealing claudins 1 and 4 is suppressed in IBD patients (104, 110), and upregulation of pore-forming claudin 2 expression and downregulation of sealing claudin 5 and 8 correlate with barrier dysfunction and active CD (111).

Disease phenotypes of human IBD are recapitulated in mice with genetic deficiencies similar to those found in human patients and confirm the importance of altered TJs and barrier integrity in IBD pathogenesis. For example, mice deficient in sealing claudin 7 in the colon develop lethal colitis soon after birth (112, 113). Notably, based on freeze fracture EM analysis, TJs of wild type and claudin 7 knockout animals have nearly identical structure, yet the character and function of the TJ appears compromised. Without claudin 7, the epithelial barrier is more permeant to small molecules (~400 Da), although the flux of larger molecules (~4 kDa) and the overall balance of Na^+ and Cl^- were unchanged (112). Therefore, claudin 7 appears to function to selectively regulate permeability of small molecules, and its dysregulation is sufficient to cause disease. The observation that loss of claudin 7 disrupts the TJ seal raised the possibility that this claudin might also limit flux of bacterial antigens. Accordingly, antibiotics ameliorated colitis in mice with claudin 7 deficiency, whereas addition of bacterial antigen (fMLF) reversed this

protective effect (112). Together, these data support a model of IBD pathogenesis in which dysregulation of TJs facilitates leakage of lumenal antigens across the epithelial barrier that trigger inflammation and initiate colitis. Master regulators of claudin expression have also been implicated in mouse models of IBD. As in humans, Hnf4α regulates expression of claudin genes in mice (114, 115). Accordingly, loss of function alleles of Hnf4α in mice results in dysregulation of claudin expression and spontaneous colitis (116).

Defective mucin production or processing has also been linked to human IBD. UC patients have fewer colonic goblet cells and decreased synthesis and secretion of MUC2, especially during episodes of severe disease. This allows direct contact of the colonic microbiota with the epithelium (117). In addition, lower levels of the goblet cell differentiation factors HATH1 and KLF4 were evident from patients with active UC (118). Furthermore, genome-wide association studies (GWAS) have implicated mutations in the *MUC* genes in IBD pathogenesis, including the membrane-anchored MUC3 and MUC19 that attach the inner mucus layer to the epithelium (99, 119). Moreover, variants of MUC2, the major secreted mucin in the human colon, have been found in IBD cases (120).

Unlike UC patients, CD patients exhibit increased mucus production, likely because the chronic inflammation leads to increases in goblet cell differentiation in the small intestine (118, 121). However, despite the increased mucus levels, the mucus barrier still fails to restrict bacterial access to the epithelia (122). These observations suggest that secondary structural modifications, in addition to mucin levels, are critical for function of the mucus barrier. In this regard, it has been proposed that increased bacterial access is due to impaired mucin processing in CD patients, which may affect the length of glycans attached by O-glycosylation (123, 124). Notably, proper glycosylation, sulfation, and sialylation are essential for the viscoelastic functions of mucus.

Humans and mice with IBD have been found to have higher levels of sulfide, a product of sulfate reducing bacteria, that could reduce the disulfide bond between mucins and degrade the mucus network, thus allowing increased microbial contact with the host (125). Accordingly, glycosylation and sulfation defects have also been found in a UC cohorts, indicating that mucus modification may play a role in limiting barrier function in this disease as well (126–128). More specifically, mutations in core 1 β3GalT specific molecular chaperone (Cosmc), a chaperone for the T-synthase glycosyltransferase responsible for the synthesis of the O-glycans on mucin proteins, have been associated with IBD in GWAS studies (129). Furthermore, the location of Cosmc on the X chromosome may provide an explanation for the male gender bias of IBD (130).

Studies in mice deficient in the Muc2 protein (*Muc2*$^{-/-}$) have helped to illustrate the importance of the mucus barrier in separating the contents of the lumen from the epithelium. These mice lack both the inner and outer mucus layer and display direct contact between the microbiota and epithelium (41, 48). Starting at five weeks after birth, *Muc2*$^{-/-}$ mice develop spontaneous colitis and display increased susceptibility to experimental DSS colitis, presumably due to the direct contact of intestinal microbiota with the epithelium (131). Animal models have also demonstrated that glycoprotein modification is crucial to intestinal homeostasis. Mice lacking core 1-derived O-glycans, recapitulating defects in humans with mutations in Cosmc, show loss of mucus complexity and rapidly develop spontaneous colitis (126).

The outer layer of colonic mucus is a habitat for both indigenous and transient microorganisms, called autochthonous and allochthonous, respectively. Changes in the resident autochthonous bacteria appear to have more impact on the host's health than do changes in transient luminal allochthonous bacteria found in the fecal matter (132). An imbalance of the microbiota, a state referred to as dysbiosis, is characteristic of IBD, indicating the importance of

maintaining the appropriate bacteria in the intestinal mucus (133). However, it is still unclear whether dysbiosis is a trigger of IBD or a result of intestinal inflammation (97). Regardless, specific members of the microbiota have been found to either worsen or ameliorate IBD. The mucus degrading bacterium *A. muciniphila* is underrepresented in many disease states including IBD, obesity, and type 2 diabetes, and numerous studies have correlated the presence of *A. muciniphila* with a healthy mucosa (134, 135). Additionally, providing mice with *A. muciniphila* during high-fat diet feeding, which normally results in decreased barrier integrity, led to a restored barrier, increased goblet cell numbers, and prevention of metabolic endotoxemia (135–137). Together, these studies demonstrate the importance of intestinal mucus in supporting growth of protective commensal bacteria as well as facilitating repopulation and maintaining commensal homeostasis to prevent dysbiosis. Accordingly, disruption of the mucus barrier may result in dysbiosis resulting in a feedback cycle of worsening disease.

Inflammaging

Aging is a complex, long-term process that affects all body systems and varies in degree between individuals. Molecular changes that occur with age, including a decline in protein homeostasis, genomic instability, and stem cell exhaustion, lead to a reduced capacity to maintain homeostatic repair and regenerative processes and eventually manifest as changes in physiological functions (138, 139). A decline in these functions is described broadly as frailty, which is an overall increased vulnerability to stressors that negatively impacts clinical outcomes in elderly patients (140). Clinical measures of frailty focus on the decline in physical capacity, as these measures best predict the likelihood of hospital admission and progression to disability (141, 142). Age related diseases (ARD), including diabetes mellitus, cancer, autoimmune diseases, and neurodegenerative

diseases, are diseases that increase in prevalence with age, especially among the frail elderly (143). Adults over 60 represent the fastest growing patient demographic world-wide. Thus, increased efforts to understand aging and frailty will become increasingly necessary to develop early detection methods and interventions that will decrease the burden of frailty and ARD in aging populations (138).

One of the most well-described changes with aging is that of the immune system. Throughout life, the immune system responds to diverse stressors, including infection and environmental exposure, with inflammation. When acute, this response helps protect against and repair damage. However, continuous low level exposure to stressors leads to accumulation of pro-inflammatory signals with age. The increase in inflammation that occurs with age, known as inflammaging, is a predictor of frailty (144). Plasma levels of the inflammatory cytokine IL-6 are undetectable in youth and begin to increase around the age of 50 (145). Higher levels IL-6 are more associated with ARD and mortality (146, 147). Additional inflammatory markers such as C reactive protein and TNFα are also observed to increase with age (143, 144, 148). In a 10-year longitudinal study of community dwelling older people (>65 years old) where frailty was measured by weight loss, weakness, exhaustion, slow movement, and low activity levels, increased risk of frailty and mortality was associated with higher white blood cell counts, including neutrophils, monocytes, and lymphocytes (149). In addition to increased baseline levels of inflammation, inducible expression of inflammatory cytokines including IL6 and TNFα in response to stimulation is more robust in aged people (150–152). However, despite increased inflammatory signals, aged phagocytes are inefficient at killing bacteria. Of clinical importance, this predisposition towards inflammation increases immunopathological complications from infections and cancer immunotherapies (152).

In contrast to increased inflammation from the innate immune system, immunosenescence is observed in the adaptive immune system with age. Features of adaptive immunosenescence include fewer B and T cell progenitors, decreased T cell proliferation in response to antigen stimulation, reduced immunoglobulin production by B cells, increased autoantibody production, and accumulation of memory $CD8^+$ T cells at the expense of naïve T cells. These changes result in impaired immune responses to infections and cancer (153, 154). The degree and rate of immunosenescence may depend on genetics. A tendency towards longer lifespan can be inherited and is associated with a lower degree of inflammaging and immunosenescence (155). Lifetime history of pathogen exposure also plays a role in immune changes. Immunosenescence may occur earlier in life in less-developed countries where people are chronically exposed to more antigens in their environment (154).

An additional hallmark of the aging immune system is decreased production of the immunoregulatory cytokine IL10 and impaired Treg function (138, 153). Lower levels of IL10 are associated with increased ARD and frailty, and people who have "successfully" aged past 100, known as centenarians, are more likely to possess a genotype that is associated with a higher IL10 production (156–158). Studies in humans pose inherent difficulties due to variability, especially after aging where the effects of a lifetime of genetic and environmental differences compound. Mice deficient in IL10 are a useful model of chronic inflammation and frailty (159, 160). Similarly to frail humans, *Il10*$^{-/-}$ mice have elevated mortality rates and display elevated IL6 levels, impaired motility, and muscle weakness earlier than age and sex-matched wild-type controls (161). Given the critical role IL10 plays in intestinal barrier homeostasis and preventing inflammatory responses, defects in IL10 signaling may contribute to inflammaging and frailty via inflammation driven by impaired intestinal barrier function. Indeed, in aged humans, IL10 levels are inversely

associated with levels of circulating bacterial muramyl dipeptide, suggesting a role for IL10 in regulating intestinal barrier function (162). The observation that germ-free mice do not display age associated inflammation unless colonized with the microbiota from conventionally aged mice furthers the association of intestinal barrier function with inflammaging. Aging is also associated with a decline in beneficial microbes that play a role in maintaining barrier function and an increase in pro-inflammatory commensal species (163). The resulting dysbiosis further fuels increases in intestinal permeability and inflammation (164).

Increased pro-inflammatory markers are seen to some degree in both frail and healthy older people (165). Therefore, it seems as if the ability of the body to cope with accumulated stress and increased inflammation, known as stress tolerance, varies among individuals (143). When stress tolerance is high, aging individuals are healthy and free from ARD for a longer period of time, and conversely, when stress tolerance is low, the individual spends more time in a state of frailty and is at increased risk for ARD. The amount of time spent in a state of health rather than a state of frailty is known as healthspan (166). Efforts to increase lifespan should occur concomitantly with efforts to increase healthspan in order to decrease the chance of spending the extra time in a state of frailty. IL10 is a potential mediator of stress tolerance with age. Manipulating IL10 levels may improve barrier function and reduce exacerbated inflammatory responses, offering a potential area for intervention to slow age-related changes of the immune system and increase healthspan.

Indoles as Therapeutics for Inflammaging

Bacteria that possess the enzyme tryptophanase A (TnaA) are capable of catabolizing dietary tryptophan into indole. The commensal microbiota of the human and mouse intestines

abundantly produce indole, which can reach concentrations of around one millimolar in the colon. Further oxidation of indole by bacterial or eukaryotic oxygenases results in a wide array of indole derivates (167). Additionally, dietary cruciferous vegetables such as brussels sprouts and kale are sources of indole-based glucobrassicin that is cleaved into indole derivates during the digestive process (168). Germ-free mice have significantly less indole in their feces, indicating the bulk of indole production is the product of bacterial metabolism (169). Our lab has identified indole and its metabolites, including indole-3-carboxaldehyde (ICA), as molecules derived from commensal bacteria that increase integrity of the intestinal epithelial barrier and thereby mitigate damage in response to various stressors, including pathogens (170), inflammatory immune responses, and environmental stressors (171). Indole also has far reaching effects on other systems outside of the intestines. We have additionally shown that indoles act across diverse phyla (*C. elegans*, Drosophila, and mice) to augment health prolong healthspan (166).

Benefits of Using Bacterial Metabolites

The importance of the intestinal microbiota in health and disease has become increasingly apparent and has led many to catalogue the types and ratios of particular bacterial species in the intestinal tract. In this regard, manipulation of the microbiota through probiotics or replacing the microbiota with fecal microbiota transplant (FMT) is becoming an increasingly popular target for disease treatments and health interventions. However, an important unresolved question is how molecules secreted by the microbiota govern interactions amongst commensals, pathogens, and the host. In theory, probiotic therapies are attractive based on the ease of directly adding desired species with known benefit to the host. However, in clinical trials, probiotics often show lackluster results. For example, while some studies have shown probiotic therapy to reduce inflammation

and increase remission rates in ulcerative colitis patients, results are inconsistent and show worsened disease in some patients (39, 172, 173). Additionally, it is difficult to introduce a new species to this environment that will be able to establish itself as a resident. And, if the probiotic strain does colonize, it is unknown what the long-term consequences would be on the other members of the microbiota and the host.

The idea of using fecal bacteria to treat disease is not new. Records of crude methods of FMT date back to fourth-century Traditional Chinese Medicine as a treatment for food poisoning, diarrhea, and fever (174). We now know that FMT involves replacing the microbiota with that of a healthy donor to allow for the healthy microbiota to restore healthy gut physiology in the recipient. By donating the entire microbiota rather than one or a few species of bacteria, the complex relationships between different members can be theoretically maintained (39). While modern FMT has been used with success for treatment of *Clostridium difficile* infection (175, 176), there is much less evidence for success in treatment of IBD and other dysbiosis linked conditions (177, 178). FMT may only lead to a transient shift in the microbiota to that of the donor (179), and since engraftment of the donor microbiota is dependent upon many factors in the recipient, including genetics, immune response, existing species of bacteria in the microbiota, diet, and lifestyle, efficacy can be difficult to predict (177).

Complex host-microbiota relationships have evolved such that many metabolites produced by the microbiota are bioactive and potentially essential or beneficial to healthy host physiology (180). The importance of bacterial metabolites to the host is supported by the idea of the 'core' adult microbiome (181). There can be variations between individuals in the species that comprise a healthy microbiome, but functionally, at the gene level, they fill the same roles (182). Tryptophan catabolism by tryptophanase is an example of a functional role of the microbiota that can be

performed by many different bacterial species (183). Probiotic therapies may not be "one size fits all," but identifying common functional roles and metabolites among probiotic species will likely improve the success of microbiota-directed therapies. Indole represents one such health-promoting bacterial metabolite that can be produced by many different bacterial species. An advantage of studying molecular products of microbiota, as opposed to cataloging the bacterial species that produce them, is that metabolites identified as protective can be immediately translated into "natural product therapeutics." Additionally, dietary manipulation to increase tryptophan may be a practical way to increase production of beneficial indole metabolites from an established microbiota.

Indoles Augment Barrier Integrity

Our group has reported that indoles augment integrity of the epithelial barrier, which limits host exposure to bacteria and bacterial antigens and systemic inflammation, via Type I IFN (IFN1) signaling. The IFN1 pathway is activated by signals from injured or damaged cells (DAMPS) and from pathogens (PAMPS), which are sensed by cytosolic RNA and DNA sensors and by toll-like receptors, which then drives production of IFNα and β. Secreted IFNα/β acts via the IFNα/β receptor in target cells to amplify the IFN response and induce IFN stimulated genes (ISGs)(184). In a mouse model of Graft vs. Host Disease (GvHD) where ICA extends survival following allogeneic bone marrow transplantation, ISGs were upregulated in response to damage, indicating activation of IFN1 signaling. ISGs were not upregulated with GvHD or ICA alone, indicating synergy between ICA and DAMPs results in IFN1 activation (171).

The initial radiation exposure to ablate recipient bone marrow damages the intestinal epithelium, resulting in presentation of intestinal allo-antigens and activation of donor T cells

which then initiate an immune response against recipient cells. T cell proliferation and secretion of IFN-γ cause apoptosis of intestinal epithelia and other tissues, ultimately resulting in barrier breakdown and sepsis as bacteria leak across the damaged mucosal barrier. Importantly, ICA accelerated repair in the small intestine and colon following TBI, as shown by reduced stem cell damage, improved barrier integrity, and accelerated crypt regeneration. The protective effect of ICA on survival following lethal TBI was eliminated in mice lacking the IFNα/β receptor. Thus, IFN1 signaling mediates protective effects of ICA (171). These data suggest that indoles act via the IFN1 system to provide protection in response to stressor-induced damage signals or pro-inflammatory conditions. Other groups have shown that indole treatment of cultured cells results in increased expression of sealing claudins, including claudin 7, and reduced expression of pore-forming claudin 2, indicating increased barrier integrity (169, 185). Additionally, treatment of germ-free mice, which have impaired tight junction expression, restores barrier integrity and protects against experimental DSS colitis (169). Together, these studies demonstrate the ability of indoles to augment intestinal barrier integrity.

Indoles Extend Healthspan

Our recent studies show that ICA treatment is associated with prolonged healthspan with and without exposure to stressors in *C. elegans,* Drosophila, and mice. One of the main indicators of healthspan we used was movement (wiggling in worms, flight height in flies, and distance moved in mice), since this mirrors what is seen in humans as they age. Clinical markers of frailty in humans measure weakness and changes in gait and activity levels (142). *C. elegans* grown on indole-producing *E. coli* K12 show an increased probability of survival than animals grown on K12Δ*tnaA*, a mutant strain that does not produce indole. No significant difference in maximal

lifespan was observed. However, a right-shift in Kaplan-Meier lifespan curves indicates a prolonged healthspan prior to death. Muscle function, measured by thrashing and pharyngeal muscle pumping, was also preserved in animals grown on K12 compared to K12Δ*tnaA*. These effects were found to be dependent on the conserved transcriptional regulator Aryl Hydrocarbon Receptor (Ahr). Similarly, geriatric mice (>2 years old) colonized with K12 show improved survival, motility, and composite health scores, including measures of weight loss, hunching, activity level, and grip strength, over a three-month period compared to mice colonized with K12Δ*tnaA* (166). Together these data suggest that indoles augment health and motility across diverse phyla, even when administered late in life. ICA and other indoles represent an attractive metabolite to investigate as therapeutics to ameliorate inflammaging.

Conclusions

The overarching hypothesis of this project is that indoles promote intestinal barrier integrity, which in turn limits systemic inflammation associated with dissemination of bacterial factors ("inflammaging") and extends healthspan (Figure 1). As discussed above, much research has shown the significance of the intestinal barrier system in preventing inflammatory responses to the microbiota. Additionally, our group has demonstrated that indoles protect against diverse inflammatory stressors and prolong healthspan (166, 170, 171). We next sought to elucidate the mechanism by which indoles can prolong healthspan, specifically in regards to the intestinal barrier system and the IL10 signaling pathway. The aims of this project are, first, to characterize the effects of indoles on the colonic barrier during homeostasis in young mice and second, to identify changes in the colonic barrier and IL10 that occur with age and assess whether indoles can restore these changes.

The research presented here furthers our understanding of how barrier integrity and dysbiosis could contribute to inflammaging and of how indoles could provide protection against frailty through regulating intestinal homeostasis and upregulating anti-inflammatory signaling pathways. Clinical trials can determine whether administration of indoles (or analogs thereof) can limit susceptibility to ARD and reduce frailty. Because frailty is a key risk factor in elderly patients, and because the elderly represent the fastest growing patient demographic world-wide, drugs that reduce frailty and preclude infirmity can have an enormous economic impact. In addition, indoles or indole-regulated genes may serve as biomarkers that predict onset of frailty and susceptibility to disease, thus paving the way to important diagnostic tools.

Figures

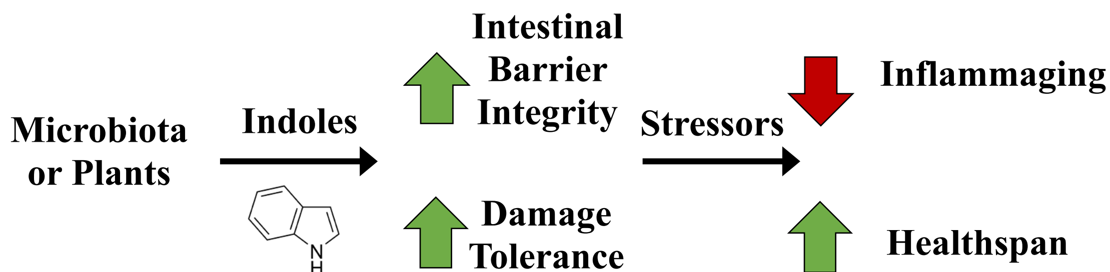

Figure 1. Indoles extend healthspan by reinforcing intestinal barrier integrity. Indoles are obtained through dietary cruciferous vegetables or from bacterial catabolism of tryptophan. Indole may be further modified into diverse indole derivatives. Indoles increase intestinal barrier integrity, which reduces inflammatory responses to intestinal antigens and increases damage tolerance of the organism. In the face of stressors, there are less exaggerated or prolonged inflammatory responses. Throughout life, accumulated exposure to stressors is thought to contribute to inflammaging. However, with increased damage tolerance, inflammaging is reduced, and healthspan is extended.

CHAPTER II

Layered Defense: How Mucus and Tight Junctions Seal the Intestinal Barrier

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Abstract

The colonic mucosa provides a vital defensive barrier separating the body from the microbial populations residing in the intestinal lumen. Indeed, growing evidence shows that loss of this barrier may cause disease or exacerbate disease progression. The loss of barrier integrity increases the translocation of bacterial antigens and stimulates inflammation in the intestinal mucosa, which is the central pathological feature of inflammatory bowel dis- eases (IBDs). This review focuses on how intestinal mucus and intercellular tight junctions (TJs) act together to maintain the integrity of the colonic barrier and how barrier integrity is dysregulated in IBD.

Introduction

Inflammatory bowel diseases (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic or intermittent inflammation of the intestinal mucosa (98). IBD is an idiopathic and multifactorial condition, and recent studies indicate that both genetic and environmental factors contribute to the disease (97, 99). During the etiology of IBD, aberrant immune responses triggered by the microbiota itself or excessive leakage of bacterial antigens into the mucosa cause inflammatory responses that progressively degrade the intestinal epithelia. This in turn permits more antigen leak and exacerbates inflammation, further compromising barrier integrity. The mucosal barrier integrity, therefore, is a key determinant of disease initiation, progression, and severity in IBD patients. Disrupting the inflammatory feedback cycle is a fundamental goal of IBD therapy. In experimental models, exposure of epithelial cells to proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) causes cell death, alters the production of secreted mucins, and disrupts the epithelial barriers) (103, 105, 106, 123, 186). Notably, the anti-TNF-α monoclonal anti- body, infliximab, a treatment for advanced IBD, short-

circuits this inflammatory feedback loop by limiting immune activation and mitigating cytotoxic effects (105).

Epithelial tissue acts not only to separate essential internal organ systems from the outside world but also facilitates communication with the microbiota and absorbs nutrients. Additional features of the colonic barrier include secreted mucus and water absorption. The epithelial layer is com- posed of a simple monolayer of around 20 billion contiguous cells (187), which, like the skin, continually regenerates. Stem cells, located at the base of the crypts of Lieberkühn, produce daughter cells that differentiate. As the progeny cells migrate toward the luminal surface, they lose their proliferative capacity and differentiate into specialized cells, which include colonocytes and mucin producing goblet cells (Figure 2) (28, 188). Cells at the luminal surface eventually undergo programmed cell death (apoptosis) and are shed (Figure 2). From birth to death, the regenerative process is complete within a few days (11).

Mature cells facing the lumen, termed surface cells, are in close apposition to layers of mucus. The cells and the mucus together form a layered barrier separating the body from the colonic microflora and limiting influx of bacteria and bacterial antigens. The combination of the epithelial sheet and the mucus layers forms a biologically flexible and environmentally responsive barrier to luminal contents. Indeed, growing evidence shows that loss of barrier integrity may cause disease or exacerbate disease progression (reviewed in (7, 38)). This review focuses on how the mucus and TJs together provide a layered defensive barrier in the colon and how their dysregulation contributes to IBD.

Mucins Separate the Epithelia from the Intestinal Lumen

The colonic mucosa secretes copious amounts of mucus, which is composed of complex and extensive O-linked oligosaccharide modifications on a mucin protein back- bone. These glycoproteins form large disulfide-linked macromolecules, and upon release from goblet cell granules into the lumen, become hydrated and expand to form a net-like gel (8). Whereas the small intestine contains a single layer of loose unattached mucus, mucus in the colon is organized into three distinct layers. Membrane-anchored mucins associated with the colonic epithelial cells form the glycocalyx. The glycocalyx gives way to a second tightly crosslinked inner layer primarily composed of the mucin protein MUC2. The outermost layer, generated by proteolysis of the inner layer, is less dense and less viscous. A more detailed description of mucin organization in the colon is reviewed elsewhere (50, 189, 190)

These mucus layers serve to separate bacteria in the lumen from the epithelia in several ways. First, the mucus layers achieve different densities, such that commensal flora or even pathogens can reside within the low density outer layer (189, 190) but are generally excluded from the high density inner layer and glycocalyx (42). The outer mucus layers contain diverse carbohydrate motifs, immunoglobulins, and other proteins that serve as binding sites for bacteria (41). A second means of separating the bacteria is mucus turnover. As secreted mucus is extruded, proteolyzed in the inner layer, and ultimately secreted into the lumen, bound bacteria are expelled and removed from the body by intestinal peristalsis. The mucus layers turn over in a matter of hours, providing dynamic removal of bacteria and limiting their access to the epithelium. Finally, the mucus serves as a lubricant to prevent feces from abrading and tearing the epithelia (49). Thus, the mucus serves to limit access of bacteria to the epithelia. It is important to note that the density of the mucus does not preclude access of secreted bacterial metabolites or toxins to the epithelia

based on size alone. Given the importance of these molecules in epithelial barrier integrity, a role for the mucus in regulating their access promises to be an increasingly important area of investigation.

Defective mucin production or processing has been linked to human IBD. UC patients have fewer goblet cells and decreased synthesis and secretion of MUC2, especially during episodes of severe disease. This allows direct contact of the colonic microbiota with the epithelial barrier (72). In addition, lower levels of the goblet cell differentiation factors HATH1 and KLF4 were evident in biopsies from patients with active UC (191). Furthermore, genome-wide association studies (GWAS) have implicated mutations in the MUC genes in IBD pathogenesis, including the membrane-anchored MUC3 and MUC19 (99, 119). Moreover, variants of MUC2, the major secreted mucin in the human colon, have been found in IBD cases (120).

Unlike UC patients, CD patients exhibit increased mucus production (121, 192). However, despite the increased levels, the mucus barrier still fails to restrict bacterial access to the epithelia (122). These observations suggest that secondary structural modifications, in addition to mucin levels, are critical for function of the mucus barrier. In this regard, it has been proposed that increased bacterial access is due to impaired mucin processing in CD patients, which may affect the length of glycans attached by O-glycosylation (72, 123). Notably, proper glycosylation, sulfation, and sialylation are essential for the viscoelastic functions of mucus. Humans and mice with IBD have been found to have higher levels of sulfide, a product of sulfate reducing bacteria, that could reduce the disulfide bond between mucins and degrade the mucus network, thus allowing increased microbial contact with the host (125). Accordingly, glycosylation and sulfation defects have also been found in a UC cohorts, indicating that mucus modification may play a role in limiting barrier function in disease (126–128). More specifically, mutations in core 1 β3GalT-

specific molecular chaperone (Cosmc), a chaperone for the T-synthase glycosyltransferase responsible for the synthesis of the O-glycans on mucin proteins, have also been associated with IBD in GWAS studies (129). The location of Cosmc on X chromosome may provide an explanation for the male gender bias of IBD (130).

Experimental disease models in mice further strengthen the role the mucus barrier and mucins can play in the prevention or pathogenesis of IBD. For example, 5 weeks after birth, Muc2 knockout animals (*Muc2*$^{-/-}$) develop spontaneous colitis and display increased susceptibility to experimental DSS colitis, presumably due to the direct contact of intestinal microbiota with the epithelia (131). Animal models also demonstrate that glycoprotein modification is crucial to intestinal homeostasis. Mice lacking core 1-derived O-glycans, recapitulating defects in humans with mutations in Cosmc, show loss of mucus complexity and rapid spontaneous colitis (126). It is important to note that the outer and inner mucus layers of the colon are almost entirely composed of Muc2, and it is expected that *Muc2*$^{-/-}$ strains would then depend only on the glycocalyx for fecal lubrication (41). In summary, the secreted mucus barrier appears to function as a means of preventing or limiting contact of bacteria and bacterial antigens with epithelial cells.

The outer layer of colonic mucus is a habitat for both indigenous and transient microorganisms, called autochthonous and allochthonous, respectively. Changes in the resident autochthonous bacteria appear to have more impact on the host's health than do changes in transient luminal allochthonous bacteria found in the fecal matter (37). An imbalance of the microbiota, a state referred to as dysbiosis, is characteristic of IBD, indicating the importance of maintaining the appropriate bacteria in the intestinal mucus (133). Mucus provides nutrients to bacteria, including amino acids and sugars, which are especially important for those bacteria capable of degrading the glycans on the mucin backbone (66, 193). *Akkermansia muciniphila* is a

mucus-degrading bacterium underrepresented in many disease states including IBD, obesity, and type 2 diabetes, and numerous studies have correlated the presence of *A. muciniphila* with a healthy mucosa (134, 135). Additionally, providing mice with *A. muciniphila* during high-fat diet (HFD) feeding, which normally results in decreased barrier integrity, led to a restored barrier, increased goblet cell numbers, and prevention of metabolic endotoxemia (135–137). Interestingly, bacteriophages within the mucus layer may also dictate the abundance and diversity of bacteria found in the intestine (133). One study of an IBD cohort demonstrated an inverse correlation between bacteriophage expansion and diversification on the one hand and bacterial diversity on the other, raising the possibility that bacteriophages may contribute to the dysbiotic state in IBD (194). Taken together, these studies demonstrate the importance of intestinal mucus in supporting growth of protective commensal bacteria as well as facilitating repopulation and maintaining commensal homeostasis to prevent dysbiosis. Accordingly, disruption of the mucus barrier may result in dysbiosis.

TJs Form a Paracellular Seal

Epithelial cells are networked together through proteinaceous adhesive contacts called junctions, which both join cells together and provide a paracellular seal. The seal between cells requires tight junctions (TJs), a specialized multipurpose adhesion that simultaneously occludes the paracellular space, dictates ion flux across the tissue, and maintains cellular polarity. The TJs are positioned at the boundary of the apical and lateral membrane surfaces of adjacent epithelial cells in the colon and consist of 5–7 membrane fusion sites called “kissing points” (29, 30). The entire circumference of each cell is joined to apposing cells via an adhesive TJ band, called a strand. TJ strands in the colon are not linear but rather highly branched structures that form

anastomosed webs that extend several hundred nanometers laterally from the apex of the cell. All epithelial cells that line the intestine are joined in this manner.

TJs regulate the flux of ions and solutes on the one hand, termed the "gate function," and maintain cell polarity on the other, termed the "fence function" (31, 32). Thus, TJs serve as a barrier to bacteria and bacterial products while also corralling apical plasma membrane proteins, and presumably the glycocalyx mucins, at the lumen-facing domain of the epithelial cell. The claudin family proteins are essential components of TJs. Claudins form TJ strands by polymerizing within the plasma membrane and dimerizing with claudins on apposing cells, across the extracellular space, to generate the paracellular seal. There are 24 claudin genes in humans, with multiple claudins expressed within any given cell (33). Importantly, several claudin proteins dimerize to form charge and size-selective ion pores that are vital for ionic homeostasis in epithelial tissues. For example, mice deficient in both claudin 2 and 15 mice fail to equilibrate sodium levels in the luminal space of the small bowel, which leads to low nutrient absorption, wasting disease, and premature death (34). Other claudins, such as claudin 4, appear to promote a tighter seal; claudin 4 does not form ion pores within the TJ but appears to exclude pore-forming claudin 2 (35). The permeability of the TJ is thought to derive at least in part from the relative amounts of amounts of pore forming or sealing claudins within the stands, as well as the architecture of the strands, particularly the complexity and numbers of TJ strands (32, 36).

Different complements of claudins are expressed at different levels in epithelia along the length of the intestinal tract, as well as within the intestinal crypts themselves (35) (Figure 2). For example, our recent studies in mice indicate that 11 claudins are expressed as a gradient within the crypts (Figure 2) (37). In general, "leaky" pore forming claudins are restricted to the colonic crypt

base, whereas "tight" sealing claudins are more prominently expressed in surface cells facing the lumen.

At the molecular level, the TJ is a highly diverse structure composed of both transmembrane and cytoplasmic proteins (195, 196). Besides claudins, there exist three additional classes of transmembrane proteins in the TJ: occludin, tricellulin, and junctional adhesion molecules (JAMs) (36, 197–199). A dense "plaque" of scaffolding molecules is anchored to the trans- membrane proteins, which include the Zonula Occludins (ZO) and MAGUK family proteins (reviewed in(98)). These scaffolding proteins link the transmembrane proteins to kinases and signaling molecules that localize at the junction. These molecules in turn control not only cell-cell contacts but also the actin polymerization machinery and contractility apparatus of apically situated actin and myosin (32). Scaffold proteins have different affinities for claudins and may regulate the types of claudins in the TJs (200). Likewise, the contractile machinery appears to regulate localization of claudins within the TJs (201–203). In summary, this molecular signaling apparatus controls claudin localization and function, and thus the permeability of the epithelial barrier.

In addition to forming ion pores, claudin strands have a poorly understood mechanism that permits small molecules to traverse the TJ, termed the paracellular "leak pathway" (38, 204). There is accumulating evidence that TJ strands themselves are dynamic and frequently break, reform, and ex- change claudin proteins in response to physiological, environmental, and pathogenic stimuli (35, 202). However, it remains to be established whether the paracellular leakage results from separation of transcellular dimers, strand breakage, or some other unknown mechanism. Interestingly, several pathogens, including both bacteria and viruses, have evolved means to traverse the paracellular junctions by disrupting claudins, or the actin structures that provide

structural integrity to the cell and the TJs (reviewed in (205)). For example, the bacterium *Clostridium perfringes* secretes a toxin that binds claudins 3 and 4, whereas Hepatitis C virus interacts with claudin 1 (205). In summary, the composition and numbers of the TJ strands, the type of claudins that compose them, their localization within the intestinal tract, and the intracellular signaling apparatus all contribute to the permeability of the intestinal barrier.

Several lines of evidence implicate dysregulation of the mucosal barrier, and of TJ architecture and claudin expression in particular, in the etiology of IBD (30, 104). GWAS studies have identified several genes that link TJ function to IBD (99, 107). Of the IBD implicated genes, one of the best understood is hepatocyte nuclear factor alpha (HNF4a) (108). HNF4a is a transcription factor involved in the maturation of colonocytes as they migrate out of the crypts. HNF4a regulates claudin expression, including claudin 7 (109). Moreover, multiple studies have demonstrated a change in TJ transmembrane proteins in IBD patients. Tricellulin, a specialized occludin-like molecule responsible for sealing the TJ at tricellular contacts, is decreased in UC (206, 207). Moreover, expression of the sealing claudins 1 and 4 is suppressed in IBD patients (104, 110). Furthermore, upregulation of claudin 2 expression and downregulation of claudin 5 and 8 correlate with barrier dysfunction and active CD (111). A more comprehensive review of the TJ and TJ-associated proteins dysregulated in IBD can be found elsewhere (206, 208, 209).

Disease phenotypes of human IBD are recapitulated in mice with genetic deficiencies similar to those found in human patients and confirm the importance of altering TJs and barrier integrity in IBD pathogenesis. For example, mice deficient in claudin 7 in the colon develop lethal colitis soon after birth (112, 113). Notably, based on freeze fracture EM analysis, TJs of wild type and claudin 7 knockout animals have nearly identical structure, yet the character and function of the TJ appears compromised. Without claudin 7, the epithelial barrier is more permeant to small

molecules (~400 Da), although the flux of larger molecules (~4 kDa) and the overall balance of Na^{+} and Cl^{-} were unchanged . Therefore, claudin 7 appears to function to selectively regulate permeability of small molecules, and its dysregulation is sufficient to cause disease. The observation that loss of claudin 7 disrupts the TJ seal raised the possibility that this claudin might also limit flux of bacterial antigens. Accordingly, antibiotics ameliorated colitis in mice with claudin 7 deficiency, whereas addition of bacterial antigen (fMLF) reversed this protective effect (112). Together, these data support a model of IBD pathogenesis in which dysregulation of TJs facilitates leakage of luminal antigens across the epithelial barrier that trigger inflammation and initiate colitis. Master regulators of claudin expression have also been implicated in mouse models of IBD. As in humans, Hnf4a regulates expression of claudin genes in mice (114, 115). Accordingly, loss of function alleles of Hnf4a in mice results in dysregulation of claudin expression and spontaneous colitis (116).

The Mucosal Barrier, Inflammation, and IBD

The mucosal barrier is not a static structure, and epithelial tight junctions and mucus production both respond to inflammatory stimuli. For example, upon infection, the inflammatory cytokine TNF-α increases epithelial permeability through alterations in TJ function, structure, and dynamics (35). Yet, TNF-α also increases mucus production by goblet cells to limit the inflammatory response by stemming influx of bacteria through the mucus layers.

Importantly, sustained inflammation or protracted dysregulation of barrier integrity initiates or exacerbates IBD. In this regard, loss or dysregulation of either the mucus or the TJs suffice to cause colitis, an effect that depends upon bacterial or antigen translocation. Thus, mice lacking claudin 7, Hnf4a, and Muc2 all develop spontaneous colitis (108, 112, 210). The

observation that symptoms are relieved under germ-free conditions or after treatment with antibiotics highlights the non-redundant role of the mucus as well as TJs in limiting access of microbes and microbial products to the body and the contribution of bacterial antigens to colitis. Additionally, the mucus barrier and TJs are interdependent such that loss of one diminishes the other. In this regard, *Muc2*$^{-/-}$ mice display increased epithelia barrier permeability and dysregulated claudin gene expression in addition to defects in the mucus (210). Likewise, numbers of mucin-producing goblet cells and mucus are diminished in *Hnf4a*$^{-/-}$ mice (116). Such interdependence could result from dysregulation of signals that coordinate both the mucus and TJs. Alternatively, inflammation associated with either knockout phenotype could damage the remaining barrier. In any case, such interdependence may facilitate feedback inflammatory responses that perpetuate IBD.

Summary and Future Directions

The epithelial TJs and the three mucus layers cooperate to form a highly integrated barrier system that together limit access of luminal contents to the body proper. The molecular constituents of this barrier are still being identified and their functions elucidated. Nevertheless, through in vivo and in vitro studies in experimental models, as well as studies of human IBD, an integrated understanding of mucosal barrier function is beginning to emerge. The capacity of the mucus to prevent abrasion and trap bacteria represents the first line of defense, while the paracellular TJ barrier prevents leakage of bacterial antigens from the lumen into the body. Furthermore, the rapid turnover of both mucus and lumen-facing epithelial cells ensures that bacteria that do interact with these structures are constantly being evicted. This multilayer system thus functions to limit bacterial contact with the host while still permitting access of small molecules, including microbial metabolites.

A comprehensive understanding of the epithelial barrier system and its relationship to commensal microbes and to the immune system may provide a more integrated approach to treatment of IBDs. For example, strategies aimed at strengthening both the mucus and epithelia barriers and thereby reducing exposure to inflammatory antigens, coupled with therapeutics that reduce susceptibility of the mucosa to damage caused by bacteria or by inflammatory responses, may restore the intestinal tract of IBD patients to a more benevolent state.

Figures

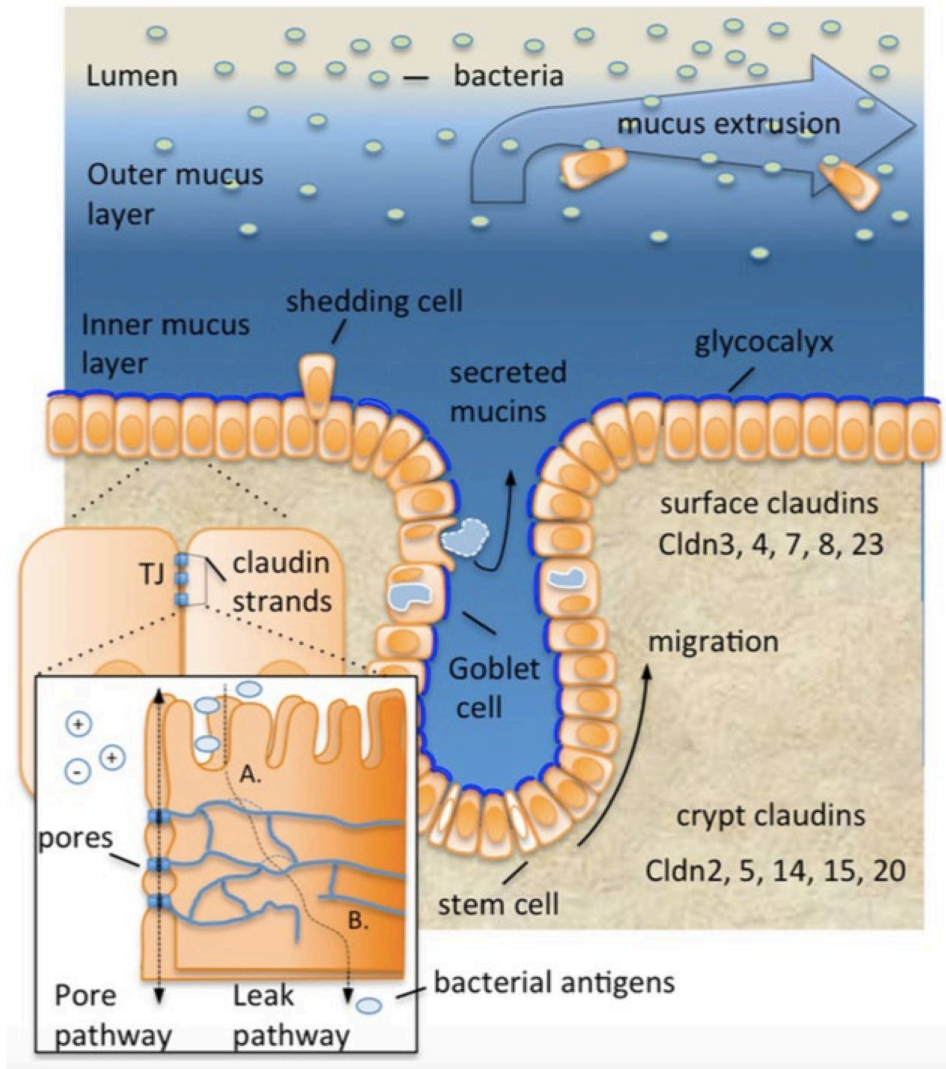

Figure 2. Barrier integrity of the colon depends on coordinated function of both epithelial and mucus barriers. The outer mucus layer, composed of Muc2 and various carbohydrate modifications, interacts with colonic microflora, while the density of the inner layer prevents bacterial penetration. The glycocalyx is attached to the plasma membrane. Together, the inner and outer mucus layer limit abrasion and trap bacteria, thereby restricting their contact with the epithelia. In general, the mucus limits contact of bacteria with underlying epithelial cells but does not restrict access of bacterial metabolites. The epithelial paracellular barrier is composed of

intercellular contacts called tight junctions (TJs). TJ strands, composed of proteins called claudins, connect apposing cells and occlude the paracellular space. Some claudins form ion pores within the TJ (pore pathway, inset), which selectively permit ion and water exchange. However, bacterial products may breach TJ defenses upon separation (a) or rupture of the TJ strands (b). Epithelial cell turnover likely helps to remove attached bacteria, and mucus flux ensures trapped bacteria are eliminated. Stem cells residing in the crypt bases produce progeny that migrate toward the lumen surface. While doing so they differentiate, producing goblet cells that secrete mucins. Differentiating cells also change the complement of claudins that they express, such that pore forming claudins are more highly expressed in the crypt-base compared to the luminal surface.

CHAPTER III

Indoles from the Commensal Microbiota Act via AHR and IL10 to Tune the Cellular Composition of the Colonic Epithelium During Aging

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Abstract

The intestinal epithelium is a highly dynamic structure that rejuvenates in response to acute stressors and can undergo alterations in cellular composition as animals age. The microbiota, acting via secreted factors related to indole, appear to regulate the sensitivity of the epithelia to stressors and promote epithelial repair via IL22 and Type I Interferon (IFN) signaling. As animals age, the cellular composition of the intestinal epithelial changes, resulting in a decreased proportion of goblet cells in the colon. We show that colonization of young or geriatric mice with bacteria that secrete indole and various derivatives or administration of the indole derivative indole-3 aldehyde (ICA) increases proliferation of epithelial cells and promotes goblet cell differentiation, reversing an effect of aging. To induce goblet cell differentiation, indole acts via the xenobiotic aryl hydrocarbon receptor AHR to increase expression of the cytokine IL10. However, indole effects on goblet cells did not depend on Type I IFN nor on IL22 signaling, pathways responsible for protection against acute stressors. Thus, indoles derived from the commensal microbiota regulate intestinal homeostasis, especially during aging, via mechanisms distinct from those used during responses to acute stressors. Indoles may have utility as an intervention to limit the decline of barrier integrity and the resulting systemic inflammation that occurs with aging.

Significance Statement

Aging is associated with an increase in baseline inflammation, a concept known as inflammaging, that may contribute to frailty in geriatric populations. Inflammaging could result from a decrease in anti-inflammatory mediators, such as IL10, or a decrease in the ability of the intestinal barrier to exclude inflammatory antigens. We show that during aging, mice lose goblet

cells, which secrete mucus to protect the epithelium. Small molecules related to indole, which are secreted by the intestinal microbiota, act via the aryl hydrocarbon receptor and the cytokine IL10 to restore depleted goblet cell numbers in aged animals. Indoles may have utility as therapeutics to protect against or reverse changes in epithelial homeostasis associated with aging, and may limit age-associated systemic inflammation, perhaps via induction of IL10.

Introduction

Aging is associated with an increase in baseline inflammation, a concept known as inflammaging, that is thought to contribute to frailty in geriatric populations (211, 212). One proposed mechanism for inflammaging is that a decline in epithelial barrier function allows for leak of antigens that results in a chronic inflammatory response (163, 164). The colonic epithelium functions as a dynamic barrier that precludes systemic access of microbial and food-derived antigens while mediating crosstalk between the intestinal microbiota and the host (46). To maintain homeostasis, intestinal stem cells (ISCs) at the base of crypts proliferate and subsequently differentiate as they migrate towards the luminal surface. Daughter cells become either absorptive colonocytes or secretory cells, including goblet and neuroendocrine cells (213). Goblet cells play a critical role in maintaining the integrity of the colonic barrier. Glycosylated mucin-2 (MUC2) proteins synthesized by goblet cells are released into the lumen where they crosslink, expand, and undergo hydrolysis to form distinct layers over the colonic epithelium. The inner mucus layer is relatively impermeable to bacteria, but porous enough to allow small molecules to reach the epithelial surface (214). The mucus also provides lubrication that limits abrasive effects of fecal matter (49). When the mucus barrier is compromised, as in *Muc2*$^{-/-}$ mice, bacteria can directly interact with the epithelium, which results in antigen and/or bacterial translocation into the lamina

propria triggering an elevated inflammatory response (41, 131). Notably, goblet cells and colonic mucus have been associated with protection against various pathogens (215–217). Moreover, a reduction in goblet cells has been associated with colitis (218).

Whereas significant information is available about the intrinsic mechanisms that control stem cell proliferation and differentiation (213), much less is known about how these processes change during aging, nor how cell signaling pathways could be targeted to ameliorate the age-dependent decline in barrier function. Recent work by us and others identified indole and its metabolites, including indole-3-carboxaldehyde (ICA), as molecules derived from commensal bacteria or from cruciferous vegetables (e.g. kale, broccoli and other *Brassica* varietals) that promote protection in response to damage by acute stressors including infection, irradiation, and hyperinflammatory allogeneic immune responses associated with Graft versus Host Disease (GvHD)(170, 171, 219). Additionally, indoles protect against a decline in health in aging mice when delivered over extended periods (weeks to months) in the absence of overt inflammatory stimuli (166).

The question arises as to how indoles orchestrate repair and immune responses so as to provide protection against diverse stressors. Indole acts via the Aryl Hydrocarbon Receptor (AHR) to induce IL22, which promotes stem-cell mediated repair of the epithelial barrier and protects against infection and damage caused by hyperinflammatory responses (219–222). Likewise, indoles induce Type I IFN signaling (171). However, induction of IL22 and IFN signaling by indoles only occurs in the context of inflammatory responses to acute stressors. These responses appear to be temporally and spatially regulated. Thus, whereas IL22 is only transiently induced in the colon, it is constitutively expressed in the small intestine and can be further induced by appropriate stimuli (223). Moreover, effects of indole appear to be context-dependent. IL22 can

induce pro- or anti-inflammatory responses depending on the disease model (224). Together, these data led us to consider the possibility that the protective responses orchestrated by indoles in response to acute inflammatory stressors may be distinct from those induced during homeostasis, including during normal aging.

Here, we show that indoles act via AHR and IL10 to alter intestinal homeostasis by augmenting intestinal cell turnover and promoting goblet cell differentiation, a process dysregulated as animals age. Our data raise the possibility of using indoles as therapeutic modalities to treat age-related infirmities associated with epithelial barrier disruption and systemic inflammation.

Materials and Methods

Animals

C57BL/6J, B6.129P2-$Il10^{tm1Cgn}$/J ($Il10^{-/-}$), B6(Cg)-$Ifnar1^{tm1.2Ees}$/J ($Ifnar1^{-/-}$) and B6.129-Ahr^{tm1Bra}/J ($Ahr^{-/-}$) mice were purchased from The Jackson Laboratory or bred in house at Emory University. Young mice were used between 8 and 12 weeks of age. Mice were allowed to acclimate in the facility for at least one week following shipment. Knockouts were house separately from wild-type controls for at least a week before and throughout experiments. Treatment groups were also housed in separate cages. Geriatric C57BL/6J mice were housed in our facility for 22-24 months. Geriatric (2 year old) BALB/c mice were obtained from the Aged Rodent Colonies maintained by the National Institute on Aging, a division of the National Institutes of Health. Animal handling and experimental protocols were in accordance with the *Guide for the Care and Use of Laboratory Animals* (225) and approved by the Emory University Institutional Animal Care

and Use Committee. IL22$^{-/-}$ mice were bred and housed at Georgia State University, Atlanta, Georgia, USA under institutionally approved protocols.

ICA Administration

ICA (Sigma) was delivered daily for 14 days by oral gavage at a dose of 150 mg·kg^{-1}·d^{-1} in a vehicle of DMSO/PEG400/5% citric acid (1:4.5:4.5).

Colonization with K12 or K12ΔtnaA

Geriatric (2 year old) BALB/c mice were given streptomycin in their drinking water (5 g/L) starting 24 h before colonization. *E. coli* K12 and K12Δ*tnaA* strains were grown to saturation at 37 °C in LB containing streptomycin (100 µg/mL) and were introduced to mice by a single oral gavage (450 µL of culture per mouse, pelleted and resuspended in 200 µL of sterile PBS) 24 h after initiation of streptomycin treatment. Mice remained on streptomycin for the duration of the experiment. Colonization levels in the gut were indirectly assessed by serial dilution plating of fecal samples on McConkey Agar containing streptomycin (100 µg/mL) and nalidixic acid (50 µg/mL). The resulting colonies were checked for indole production using Kovacs reagent (Sigma) following overnight growth in LB at 37 °C.

AB-PAS Staining for Goblet Cell and Mucus Quantitation

A 1cm piece of colon around the most distal fecal pellet in the colon was excised and fixed in Methacarn solution (60% methanol, 30% chloroform, 10% glacial acetic acid) for 1 week. Fixed tissue was processed by two washes each of methanol, 100% ethanol, and xylene substitute for 30 minutes, 20 minutes, and 15 minutes respectively. The tissue was transferred to the Winship

Research Pathology Core Lab at Emory University for paraffin embedding, sectioning, and staining with Alcian Blue and Periodic Acid Schiff (AB-PAS). Goblet cells were quantified by counting positive cells per crypt in twenty crypts per section, two sections per mouse. Special care was taken to ensure that only full crypts were counted, where the base of the crypt and cells up to the lumen were visible and linear. For mucus thickness measurements, 10 measurements per section were made in two sections per mouse. Microscopy was performed using a Nikon Eclipse 50i with NIS Elements D Software for imaging and crypt measurements.

Detection of Muc2 Immunofluorescence

Slides of methacarn fixed tissue were dewaxed by incubation on a slide warmer at 60°C for 10 minutes followed by incubation in xylene substitute (Citrisolv) twice for 10 minutes. Sections were rehydrated in decreasing concentrations of ethanol (100, 95, 70, 50, 30%) for 5 minutes each. Antigen retrieval was performed by microwaving slides in antigen retrieval solution (10 mM citric acid, pH 6.0) for 5 minutes at 300W thrice and then left at room temperature for 30 minutes. Slides were blocked (5% FBS in PBS) for 30 minutes at room temperature and then incubated with Muc2 (1:200 in blocking solution) overnight at 4°C. Slides were washed with PBS thrice for 10 minutes each before anti-rabbit FITC secondary antibody (Jackson; 1:100) was applied for 2 hours at room temperature. Finally, DAPI (1:1000) was applied for 5 minutes followed by three 10 minute washes in PBS and mounting in Vectashield.

Detection of Ki67, pHH3(Ser10), and CgA by Immunofluorescence

Distal colon tissue was excised and fixed in 4% paraformaldehyde for 24 hours prior to processing, embedding, and sectioning by the Winship Research Pathology Core Lab (Emory

University). Slides were dewaxed by incubation on a slide warmer at 60°C for 10 minutes followed by incubation in xylene substitute (Citrisolv) twice for 10 minutes. Sections were rehydrated in decreasing concentrations of ethanol (100, 95, 70, 50, 30%) for 5 minutes each. Antigen retrieval was performed by microwaving slides in antigen retrieval solution (10 mM citric acid, pH 6.0) for 5 minutes at 300W thrice and then left at room temperature for 30 minutes. Slides were permeabilized in 0.1% Triton-X in PBS for 5 minutes and then blocked (0.1% Triton-X, 10% donkey serum in PBS) for 1 hour at room temperature. Primary antibody (Ki67 1:500; pHH3 1:1000, CgA, 1:400) in blocking solution was applied overnight at 4°C in a humid chamber. Slides were washed three times for 15 minutes in 0.1% PBS-T followed by anti-rabbit Cy3 secondary antibody (Jackson; 1:100) incubation in a dark humid chamber for 2 hours at room temperature and DAPI (1:1000) for 5 minutes. Finally, slides were washed 3 times for 15 minutes in PBS-T and mounted in Vectashield. Positive cells were counted in 20 sections per crypt.

Fluorescence Microscopy

Immunofluorescent images were acquired with a scientific-grade cooled charge-coupled device (Cool-Snap HQ with ORCA-ER chip) on a multi-wavelength, wide-field, three-dimensional microscopy system (Intelligent Imaging Innovations) (Denver, CO), based on a 200M inverted Zeiss microscope using a 20x lens with a numerical aperture of 0.5 lens (Carl Zeiss, Thornwood, NY). Immunofluorescent samples were imaged at room temperature by using a standard Sedat filter set (Chroma Technology, Rockingham, UT).

Detection of IL22 by ELISA

Two centimeter segments of distal colons and terminal ileum were excised, washed in HBSS supplemented with penicillin and streptomycin, and cut into 1 cm segments. These segments were cultured for 24 h in 24-well flat-bottom culture plates in RPMI 1640 medium supplemented with penicillin and streptomycin. Supernatant was collected and centrifuged at 5,000 g for 10 minutes at 4°C. The concentration of IL22 was measured according to the ELISA kit (Invitrogen).

RNA Sample Preparation and qPCR for Detection of Il10 Transcript

One centimeter segments of transverse colon were excised and put directly into TRI Reagent (Ambion) on ice. Tissue was homogenized in Bullet Blender Green Eppendorf lysis tubes using a Bullet Blender Storm following manufacturer's protocol for intestine samples. RNA was extracted using the TRI Reagent manufacturer's protocol. Reverse transcription was performed using RevertAid First Strand cDNA Synthesis kit (Thermo) with the oligo(dT)18 primer. qPCR was performed using iQ SYBR Green Supermix on a *MyiQ* Real time PCR system (Biorad). Delta-delta Ct analysis (ΔΔCT) method was used to quantify relative gene expression compared with *Gapdh* and *B-actin* controls, using following primers: *Gapdh* forward (5' AGG TCG GTG TGA ACG GAT TTG 3'), *Gapdh* reverse (5' TGT AGA CCA TGT AGT TGA GGT CA 3'), *B-actin* forward (5' GGC TGT ATT CCC CTC CAT CG 3'), *B-actin* reverse (CCA GTT GGT AAC AAT GCC ATG T 3'), mouse *Il-10* forward (5' GCT CTT ACT GAC TGG CAT GAG 3'), mouse *Il-10* reverse (5' CGC AGC TCT AGG AGC ATG TG 3'). The data generated by qPCR assays were normalized using the average value of the vehicle treatment control group.

Statistical Analysis

Significance was assessed using Graph Pad Prism 7 software. Comparisons were made using a Mann-Whitney, Kruskal-Wallace analysis of variance (ANOVA) with Dunn's multiple comparison's test, or Two-Way ANOVA. Nonparametric tests are appropriate because for most processes under study here, because the distribution of the data remains unknown. Rank sum tests calculate exact probabilities for the null hypothesis, and we report the probability of obtaining the observed distribution of ranked data by chance. Thus a p=0.01 or p=0.15 mean that the observed ranking of values would be observed only one or fifteen times, respectively, in 100 repeats of the experiment. In most cases, there exists enough signal to noise to keep the value well below the 0.05 convention for significance. But, with variant data, interpretation of significance and import is based on the sample size, the meaningful effect size, as well as other considerations such as relevant prior evidence, plausibility of mechanism, number of repeats, uncertainty, and variation in effect size.

Results

ICA Increases Homeostatic Stem Cell Turnover in the Murine Colon

To assess the effects of exposure to indoles in the absence of stressors, we administered ICA or vehicle to young mice (8-12 weeks) once daily for 2 weeks by oral gavage. Proliferation of cells in the crypts, as measured by the ratio of Ki67-positivity to the total number of DAPI-positive nuclei per crypt was measured (Fig. 3.1D). ICA did not change the number of DAPI-positive cells per crypt (Figs. 3.1A-B) and nor did it cause substantial hyperplasia, as measured by crypt length, which marginally increased (Fig. 3.1C). In vehicle-treated mice, Ki67-positive cells

comprise on average a third of cells at the base of the crypt. With ICA, the Ki67-positive zone encompassed half of the crypt (Figs. 3.1A,D). In accordance with these data, ICA also increased the number of cells staining positive for phospho-histone H3 (pHH3), a marker of cells transiting through G2 and M phase of the cell cycle (Figs. 3.1E,F). Thus, protracted ICA treatment increased cell proliferation but not total cell number in the colonic crypts. Together, these data suggest that ICA induces a faster rate of turnover of cells in the crypt, although other less likely possibilities exist.

Exposure to ICA Increases the Proportion of Goblet Cells in the Mouse Colon

To determine whether ICA altered the cellular composition of the crypts, histochemical or immunofluorescent staining was carried out on sections derived from young animals treated for one or two weeks with ICA. Goblet cells were detected by staining with Alcian Blue and Periodic Acid Schiff (AB-PAS; Fig. 3.1G), which recognizes glycosylated mucin, or by staining using an antibody against MUC2 (Fig. 3.1H). Whereas exposure to ICA for one week did not produce significant changes in numbers of goblet cells per crypt, exposure for 2 weeks resulted in robust increase ($p<.0001$; Fig. 3.1I). This increase represented an increased proportion of goblet cells per crypt (Fig. 3.1J) because no changes in total cell number were evident (Figs. 3.1B). By contrast, no change was evident in the number or proportion of enteroendocrine cells, detected by chromogranin A (CgA) staining (Figs. 3.1K,L). The increase in proportion of goblet cells persisted for at least one week after ICA was removed (Fig. 3.1M). Together, these data indicate that protracted exposure to ICA induces a durable increase in the proportion of goblet cells in the colonic crypts.

Indoles Restore Depleted Goblet Cell Numbers in Geriatric Mice

To assess the effect of indoles on changes in intestinal homeostasis that occur during aging, geriatric C57Bl/6 mice (22-24 months old) were administered vehicle or ICA for two weeks. Histological assessment indicated goblet cell numbers per crypt were lower in vehicle-treated geriatric mice in comparison to vehicle-treated young mice (Fig. 3.2A; compare squares in Fig. 3.2B). Accompanying this effect was a concomitant reduction in thickness of the inner mucus layer (compare squares in Fig. 3.2C). When geriatric mice were treated with ICA for two weeks, goblet cell numbers per crypt increased to a level comparable to that seen in young animals with or without ICA (Figs. 3.2A,B), though no increase in mucus thickness was evident (Fig. 3.2C). Ki67 staining in geriatric animals appeared similar to that seen in young mice (compare 3.2D with 3.1A, and 3.1D with 3.2F). ICA treatment increased proliferation as measured by an increase in proportion of Ki67-postive cells within crypts (Figs. 3.2D,F). Although there was some variance in the values, the numbers of DAPI$^+$ cells per crypt appeared slightly decreased with ICA treatment, though the numbers and variance were comparable to those from young animals (compare Figs 3.2D and 3.1B), perhaps indicating a slight difference in turnover rate in geriatric animals. Thus, aging reduced the proportion of goblet cells within the crypt, and ICA treatment restored goblet cell numbers to a level comparable to that seen in young animals.

We next determined whether sustained colonization of the intestinal tract with commensal bacteria that produce indoles would also increase goblet cell numbers in geriatric mice. To do this, geriatric (two-year old) Balb/c mice were treated with streptomycin to reduce numbers of commensal bacteria and then colonized with streptomycin resistant strains of either *Escherichia coli* K12, which utilizes tryptophanase to convert tryptophan into indole and various indole derivatives including ICA, or, alternatively, *E. Coli* K12Δ*tnaA*, a mutant strain lacking the

bacterial tryptophanase gene that produces neither indole nor indole derivatives. Upon colonization, these strains reached levels of 10^8 CFU per gram of feces and comprised the vast majority of culturable bacteria within the lumen. Animals remained colonized for up to three months, the longest time tested. Finally, animals colonized with K12 expressed significantly higher levels of the indole metabolite indoxyl sulfate in the urine compared to animals colonized with K12Δ*tnaA*, verifying increased indole production by the intestinal microbiota in the K12-colonized mice (166). We had previously characterized this same group of animals and determined that ICA improved various measures of health, including motility, aggregate healthscores, and weight over a three month period (166).

We compared goblet cell numbers in animals colonized for three months with K12 versus K12Δ*tnaA* (Fig. 3.2G). Although there appeared to be an increasing trend, a comparison of average goblet cell numbers per crypt was quite variable (Fig. 3.2H) because significant variation in the length of crypts was apparent in sections from different animals (Fig. 3.2I). When average numbers of goblet cell were normalized to average crypt length in the same animal (Fig. 3.2J), a highly significant increase in proportion of goblet cells became apparent in animals colonized with K12 as compared to K12Δ*tnaA* (Fig. 3.2J). Taken together, these data demonstrate that the indole effect on goblet cell differentiation did not depend on the mouse strain, and that indoles either delivered exogenously or produced by the commensal microbiota can alter the cellular composition of the intestinal epithelium during aging.

Effects of ICA Depend on AHR but not IL22 nor Type 1 Interferons

Indole and related molecules, including ICA, are ligands for AHR (31). To determine whether the effects of ICA on cell proliferation and goblet cell differentiation depend on AHR,

Ahr-deficient mice (*Ahr* $^{-/-}$) or wild-type littermates were treated with ICA for 2 weeks. No significant increase in goblet cell numbers was detected in *Ahr* $^{-/-}$ mice compared to WT littermates (Figs. 3.3A-B). Crypt length in *Ahr* $^{-/-}$ mice was not significantly different from that of wild-type mice and was unchanged following ICA treatment (Fig. 3.3C). Additionally, no increase in cell proliferation was evident in *Ahr* $^{-/-}$ mice following ICA treatment (Figs. 3.3D-E). Together, these data suggest that ICA effects on cell proliferation and on goblet cell numbers depended upon AHR.

Activation of AHR in type 3 innate lymphoid cells by indoles induces transient expression of IL22 in the context of various stressors (220, 223). We next determined the extent to which two-week treatment with ICA induced IL22 levels in the colon and the small intestine in the absence of stressors. IL22 was constitutively expressed in the small intestine of untreated mice; however, no detectable increase in IL22 was evident following ICA treatment in either small intestine or colon, and IL22 levels remained below the limit of detection in colons of untreated or ICA-treated animals (Fig. 3.3F). Furthermore, *Il22*-deficient mice (*Il22* $^{-/-}$) still showed an increase in goblet cell numbers with 2 week ICA treatment (Figs. 3.3G,H). These data provide evidence that exposure to ICA over protracted periods and in the absence of stressors does not induce IL22. These data further suggest that IL22 did not contribute to ICA-mediated increase in cell proliferation and proportion of goblet cells.

Type I IFN have also been shown to mediate protective responses of ICA in response to radiation and inflammation associated with GvHD (171). To test whether the effects of ICA on goblet cells depended on Type I IFN signaling, *Ifnar1* $^{-/-}$ mice, which lack the Type I IFN receptor, were exposed to ICA for 2 weeks. Goblet cell numbers in *Ifnar1* $^{-/-}$ mice increased to the same extent as in ICA treated wild-type animals, suggesting that effects of ICA on goblet cells did not

depend on Type I IFN signaling (Figs. 3.3I,J), in accordance with published data showing that indoles only induced a Type I IFN response under acute stressor conditions (171).

Effects of ICA are Mediated by IL10

IL10 has been implicated in regulating intestinal homeostasis (74, 226). To assess whether effects of ICA on cell proliferation and goblet cell differentiation depend on IL10, *Il10* transcript levels were measured by qPCR in young and geriatric animals. In response to ICA, *Il10* transcript levels increased in the colons of young mice by 2.5 fold on average (Fig. 3.4A). Differences in transcript levels in geriatric mice were less robust, but half the ICA-treated animals expressed levels of IL10 higher than all vehicle-treated animals (Fig. 3.4B). In addition, no difference in *Il10* transcript levels were evident in colons from *Ahr* $^{-/-}$ mice (Fig. 3.4C).

To determine whether the effects of ICA depended upon IL10, young *Il10*-deficient (*Il10*$^{-/-}$) mice were treated with ICA for two weeks. No increase in the numbers of DAPI^{+} cells per crypt (Fig. 3.4D), nor in crypt length (Fig. 3.4E) were evident with ICA treatment in IL10$^{-/-}$ mice. In contrast to wild type animals, no increases in goblet cell numbers per crypt (Figs. 3.4E,F), in cell number per crypt, or cell proliferation (Figs. 3.4G-I) were evident in IL10$^{-/-}$ animals. Together, these data suggest that the effects of treatment with ICA on cell proliferation and goblet cell differentiation depend on IL10.

Discussion

The capacity of the intestine to regenerate via proliferation and differentiation of crypt stem cells remains vital for repairing damaged epithelia and facilitating anti-pathogen responses. Several lines of evidence indicate that indoles from intestinal microbiota can alter responses to

acute stressors (167). Indole derivatives such as ICA can induce IL22 via AHR, which initiates an epithelial repair program (223). Additionally, indoles induce Type I IFN in the context of radiation exposure and GvHD (171). However, data presented here indicate that neither IL22 nor Type I IFN signaling function in the context of homeostatic changes evident upon protracted exposure to indoles (Figs. 3.3I,J), raising the possibility that indoles regulate responses to stressors and homeostasis via distinct mechanisms (Fig. 3.4J). Here, we show that protracted exposure to indoles alters the homeostatic setpoint within the crypts by increasing the proportion of goblet cells and promoting turnover of cells in the crypts (Fig. 3.1I). Moreover, we provide a mechanism whereby indoles regulate homeostasis, particularly as animals age, involving AHR and IL10, a cytokine dispensable for acute infections (227). Our results are in accordance with a recent *in vitro* study where the indole derivative indole-3-carbinol promoted goblet cell differentiation in enteroids via AHR (228).

It has been proposed that with age, the intestinal barrier becomes more permeable, and leakage of bacterial antigens from the lumen into the body cavity precipitates a systemic inflammatory response, a process called inflammaging (3, 4, 31). However, evidence in support of inflammaging has remained somewhat limited. Diminishing numbers of goblet cells and thinning of the mucus layer during aging could lead to altered host-microbe relationships and increased exposure to circulating luminal antigens, and, consequently, increased inflammation (229, 230). Additionally, reduced mucus production could contribute to constipation, a common complaint among the elderly (231), and permit abrasion by feces, leading to a damaged epithelial barrier and inflammation. Finally, while a decline in proliferation was not evident in geriatric mice, increased turnover associated with indoles could provide significant benefit in the event of injury or infection. Indoles and IL10 likely influence multiple aspects of the intestinal barrier to protect

against bacterial translocation and limit inflammatory responses under homeostatic conditions. In addition to increasing goblet cell differentiation, indoles also improve transepithelial resistance (90, 171) and regulate expression of the IL10 receptor (IL10R) on intestinal epithelial cells, perhaps as a means of further promote IL10 signaling (90). Together, these observations suggest that indoles can protect the epithelial barrier, especially during aging, and limit inflammation associated with transit of bacterial products.

We previously reported that colonization of geriatric mice with indole-producing bacteria for three months augmented lifespan and reduced frailty, including measures of overall healthscore, weight loss, and mobility, though the mechanistic basis for this protective effect was not elaborated (166). Here we demonstrate that in aged animals, reduced health scores are accompanied by loss of goblet cells in animals colonized with K12ΔtnaA, an effect reversed by colonization with K12 bacteria, which produce indoles (Fig. 3.2B). It is unlikely that goblet cells alone contribute to increased healthspan with indoles. However, our observation that Indole can act via IL10 (Fig. 3.4) may provide an important link.

One interesting possibility is that activation of IL10 by commensal microbiota can both initiate protective reorganization of the epithelial barrier via upregulation of goblet cells and other protective mechanisms, and simultaneously limit systemic inflammatory responses to bacterial antigens, supporting the idea that indoles are multifaceted microbiota-derived factors that limit inflammaging. In this regard, IL10 is well recognized for its anti-inflammatory activity and decreases with age (148). Moreover, mice deficient in *Il10* exhibit increased inflammation throughout their lives and are increasingly susceptible to inflammatory conditions as they age, including colitis (74, 159). Given the increased baseline inflammation, *Il10*$^{-/-}$ mice exhibit increased signs of frailty, including decreased mobility and early mortality (232, 233).

Accordingly, in a study of frail geriatric humans, amounts of circulating bacterial muramyl dipeptide was inversely correlated with IL10 levels (162). As noted above, IL10 induced by indoles may certainly protect against the onset of frailty during aging by limiting decline in barrier function that would permit translocation of bacterial products from the lumen into the lamina propria. In this regard, we have shown previously that ICA treatment of allo-BMT recipients leads to reduction of colonic inflammation, IL6, and bacterial leakage into the mesenteric lymph nodes (171). ICA also induces protective systemic responses over longer periods of time. Thus, ICA increases survival of allo-BMT recipients even when discontinued, and induces tolerance in allogeneic T cells (171). Whether such systemic effects depend on IL10 is currently under investigation.

Because dysbiosis has been associated with deleterious changes with age (212), our future studies are aimed at measuring levels of indole or its derivatives in the colons of geriatric human subjects. In this context, indole levels may provide a predictive metric of health potential. In prospective bone marrow transplantees, Indole levels predict susceptibility to intestinal GvHD and mortality (234), a metric we are using to identify prospective patients for a clinical trial. In conclusion, further delineation of the signaling pathways controlled by indoles, both within the intestinal epithelia and systemically, will facilitate development of therapeutics that limit detrimental changes associated with aging or other inflammatory diseases and promote healthspan, the capacity to live better for longer.

Figures

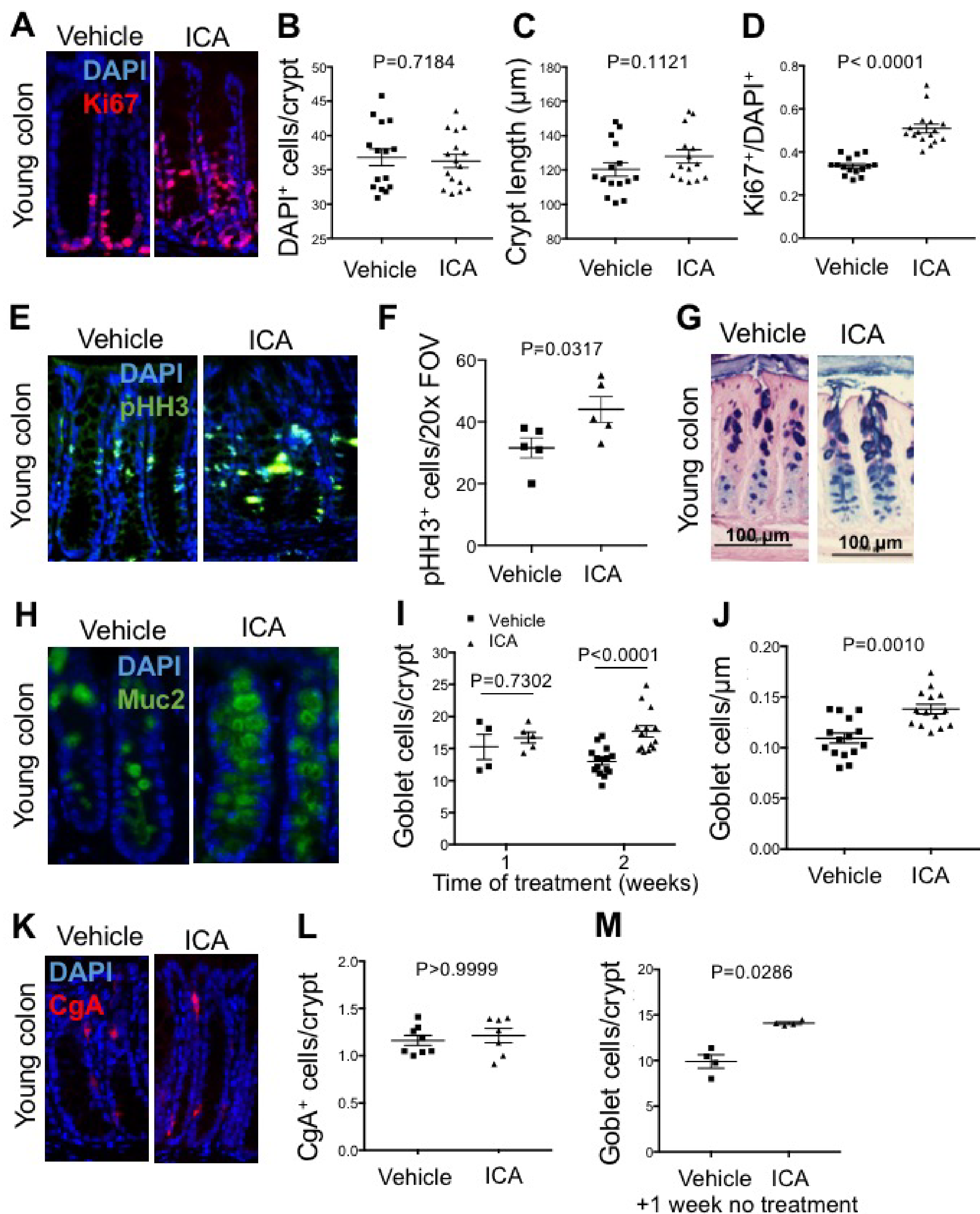

Figure 3.1 ICA increases homeostatic stem cell turnover and goblet cell differentiation in the murine colon. Two to three month old wild type C57BL/6 mice were administered vehicle or 150

mg/kg ICA once daily (QD) for one or two weeks as indicated (*A*-*L*), or two weeks with ICA followed by one week without treatment (M). (*A*) Representative images of DAPI and Ki67 immunostaining of distal colon sections. (*B*) Quantitation of DAPI-positive cells per crypt. (*C*) Quantitation of average colon crypt length. (*D*) Quantitation of number of $Ki67^+$ cells per crypt normalized to the number of $DAPI^+$ cells per crypt. (*E*) Representative images of DAPI and phospho-histone H3 (pHH3) immunostaining of distal colon. (*F*) Quantitation of number of $pHH3^+$ cells per field of view (FOV). (*G*) Representative images of Alcian-blue Periodic Acid (AB-PAS) staining of cells in distal colon. (*H*) Representative DAPI and MUC2 immunostaining of cells in distal colon. (*I*) Quantitation of AB-PAS+ (goblet) cells per crypt following one or two weeks of ICA treatment. (*J*) Quantitation of AB-PAS+ (goblet) cells per crypt, normalized to crypt depth. (*K*) Representative Chromogranin A (CgA) immunostaining (enteroendocrine cells) in distal colon. (*L*) Quantitation of CgA-positive cells per crypt. (*M*) Quantitation of AB-PAS+ (goblet) cells in distal colon in animals treated with ICA for 2 weeks followed by no treatment for 1 week. *Statistics:* Each point represents an individual mouse. Twenty full crypts per section and two sections per mouse were assessed for Ki67, AB-PAS, and CgA staining. Ten fields of view containing full crypts were assessed per section for pHH3. Data shown are representative of at least two independent experiments. Data expressed as mean ± SEM. A Mann-Whitney test was performed to calculate *P* values.

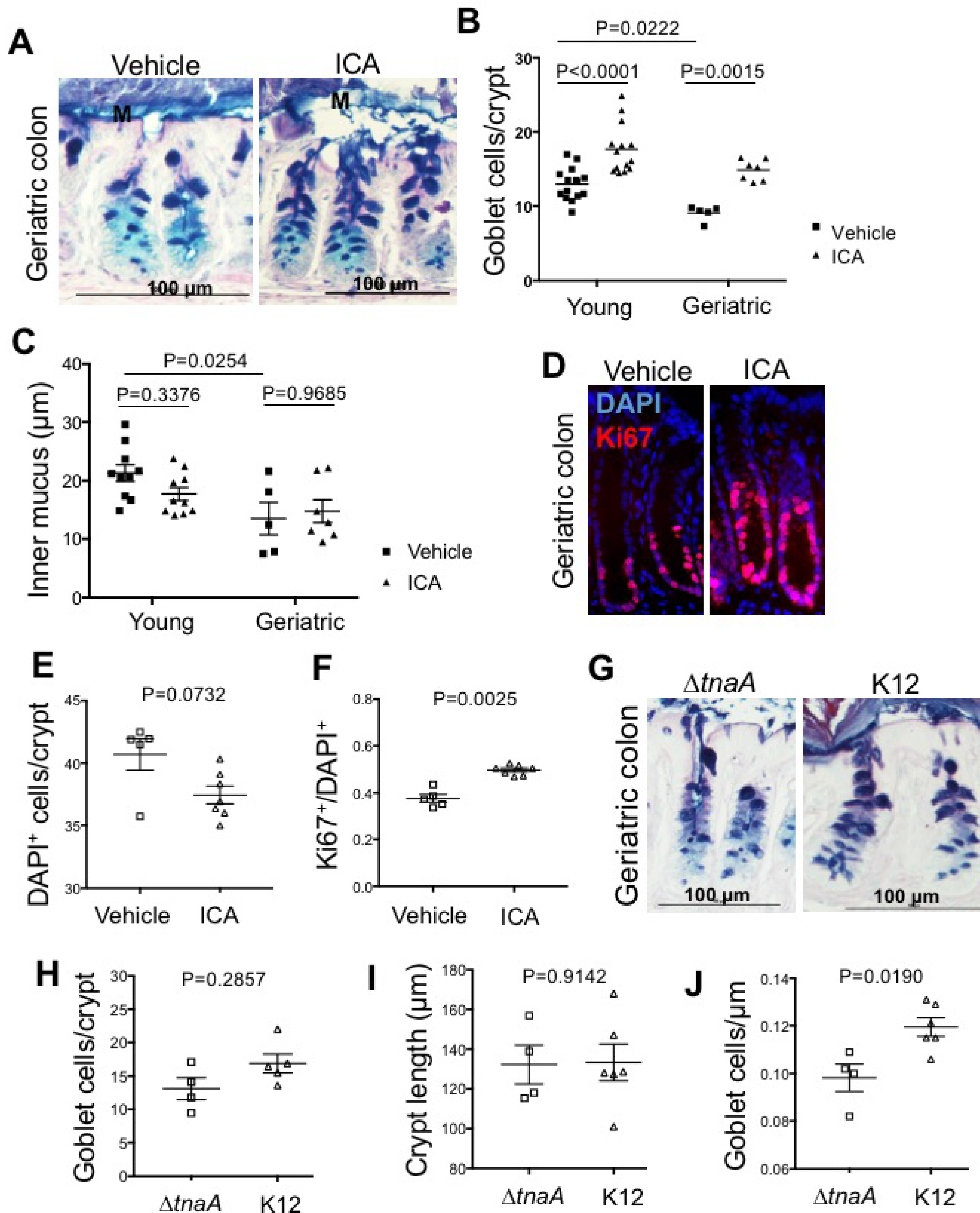

Figure 3.2 ICA restores depleted goblet cell numbers in geriatric mice. (*A*-*F*) Geriatric (22-24 months old) C57BL/6 mice were administered vehicle or 150 mg/kg ICA for 2 weeks by oral gavage. (*A*) Representative images of AB-PAS staining (goblet cells) in distal colon of geriatric

mice. "M" indicates the inner mucus layer. (*B*) Quantitation of AB-PAS staining (goblet cells) per crypt in young or geriatric mice. (*C*) Measurement of inner mucus layer thickness in young or geriatric mice. (*D*) Representative images of Ki67 immunostaining in distal colon of geriatric C57BL/6 mice. (E) Quantitation of DAPI-positive cells per crypt in geriatric C57BL/6 mice. (*F*) Quantitation of Ki67-positive cells per crypt normalized to DAPI-positive cells per crypt in geriatric C57BL/6J mice. (*G-J*) Geriatric two-year old Balb/c mice were treated with streptomycin and colonized with streptomycin and nalidixic acid-resistant K12 or K12Δ*tnaA* for 3 months before histological assessment. (*G*) Representative AB-PAS staining (goblet cells) in distal colon of colonized geriatric Balb/c mice. (*H*) Quantitation of goblet cells per crypt in colonized geriatric Balb/c mice. (*I*) Average colon crypt length of geriatric Balb/c mice. (*J*) Goblet cell numbers per crypt normalized to crypt length of geriatric Balb/c mice. Statistics: Each dot represents an individual mouse. Twenty full crypts counted per section. For mucus thickness, ten measurements were made per section. Data expressed as mean ± SEM. Two-way ANOVA with multiple comparisons (*B*,*C*) or Mann-Whitney tests were performed to calculate *P* values.

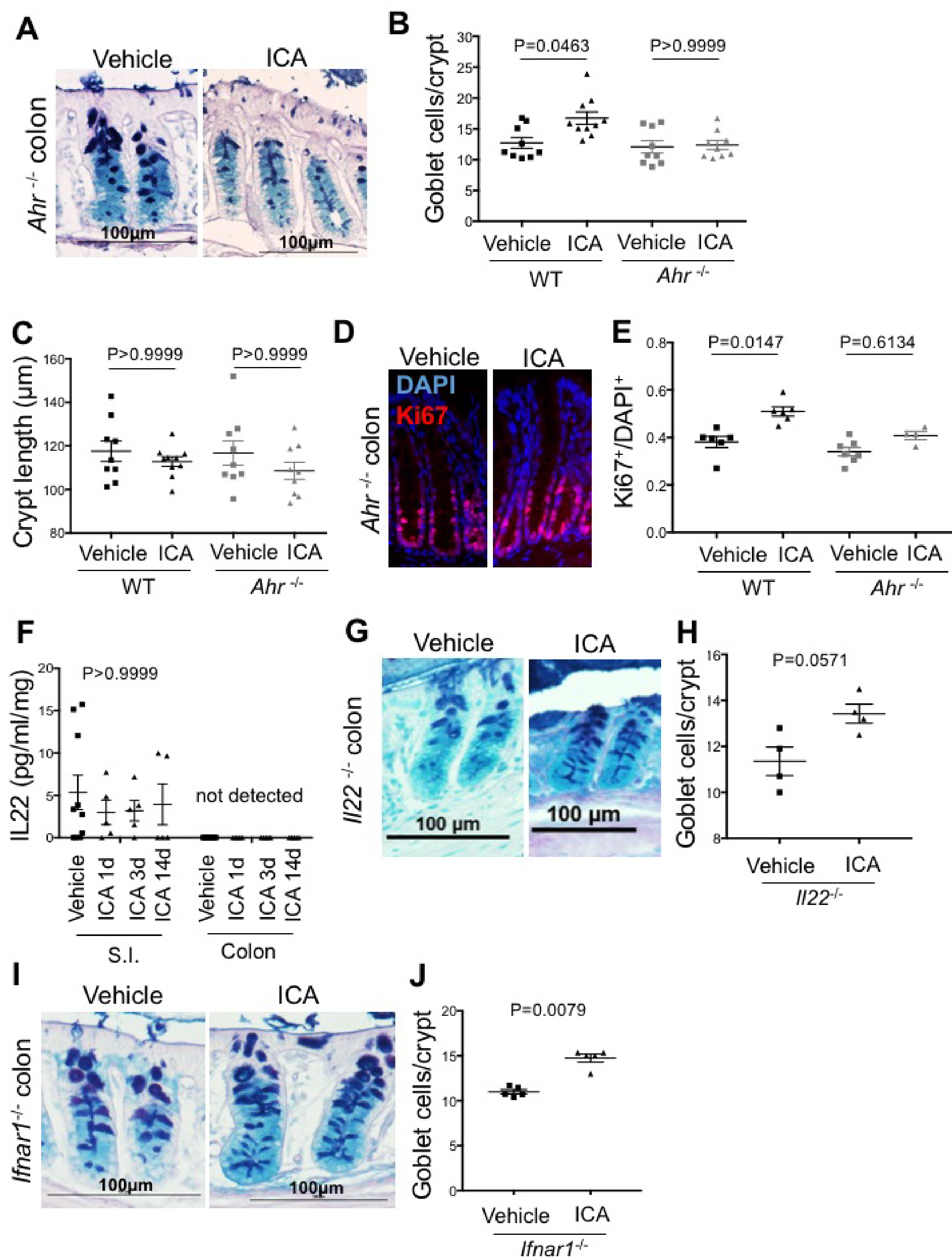

Figure 3.3 Effects of ICA depend upon AHR but not IL22 or Type I IFN. (*A-E*) Male and female *Ahr*$^{-/-}$ mice and WT littermates were administered vehicle or 150 mg/kg ICA for 2 weeks.

Data shown are pooled results from two independent experiments. (*A*) Representative images of AB-PAS staining (goblet cells) in distal colon of *Ahr*$^{-/-}$ mice treated with vehicle or ICA for 2 weeks. (*B*) Quantitation of goblet cells per crypt in *Ahr*$^{-/-}$ mice or WT littermates. (*C*) Average colon crypt length of wild type (WT) and *Ahr*$^{-/-}$ mice. (*D*) Representative images of Ki67 immunostaining of distal colons of *Ahr*$^{-/-}$ mice. (*E*) Quantitation of Ki67-positive cells per crypt, normalized to DAPI-positive cells per crypt. (*F*) Wild-type C56BL/6 mice received daily oral gavage with vehicle or 150 mg/kg ICA for 2 weeks. Two-cm pieces of distal colon or small intestine (s.i.) were excised and cultured *ex vivo*, and conditioned supernatant was collected for IL22 ELISA. (*G-H*) *Il22*$^{-/-}$ mice received daily oral gavage with vehicle or 150 mg/kg ICA for 2 weeks. (*G*) Representative images of AB-PAS staining (goblet cells) in distal colon of *Il22*$^{-/-}$ mice. (*H*) Quantitation of goblet cells per crypt. (*I-J*) *Ifnar1*$^{-/-}$ mice received daily oral gavage with vehicle or 150 mg/kg ICA for 2 weeks. (*I*) Representative images of AB-PAS staining (goblet cells) in distal colon of *Ifnar1*$^{-/-}$ mice treated with vehicle or ICA. (*J*) Quantitation of goblet cells per crypt. Statistics: Each dot represents an individual mouse. Twenty full crypts counted per section for AB-PAS stains. Data expressed as mean ± SEM. Kruskal-Wallis with Dunn's multiple comparisons (*B*, *C*, *E*, *F*) or Mann-Whitney (*H*,*J*) tests were performed to calculate *P* values.

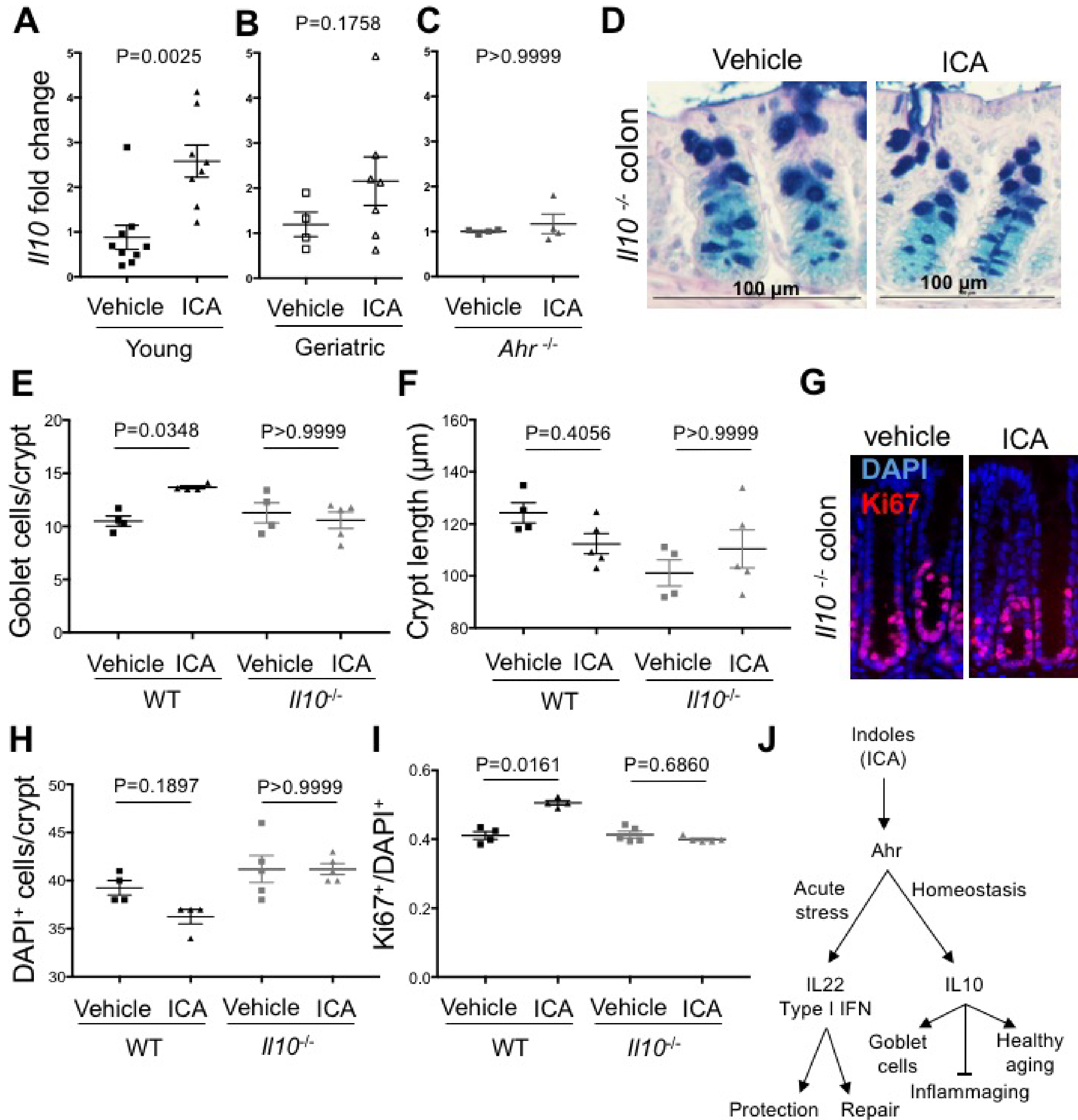

Figure 3.4. Effects of ICA are mediated by AHR and IL-10. Wild type, *Il10*$^{-/-}$ or *Ahr*$^{-/-}$ mice were treated with vehicle 150mg/kg ICA for two weeks. (*A-C*) qPCR analysis of expression of *Il10* in colon tissue of (*A*) young WT C56BL/6 mice, (*B*) geriatric (22-24 month old) WT C57BL/6 mice, or (*C*) *Ahr*$^{-/-}$ mice. (*D-I*) Measurement of goblet cells, proliferation, and cell numbers in *Il10*$^{-}$

$^{/-}$ mice or WT controls. (*D*) Representative images of AB-PAS stain (goblet cells) in distal colon of *Il10*$^{-/-}$ mice. (*E*) Quantitation of goblet cells per crypt in wild type (WT) and *Il10*$^{-/-}$ animals. (*F*) Average colon crypt length in wild type (WT) and *Il10*$^{-/-}$ animals. (*G*) Representative images of Ki67 immunostaining of distal colons of *Il10*$^{-/-}$ mice. (*H*) Quantitation of DAPI-positive cells per crypt in wild type (WT) and *Il10*$^{-/-}$ animals. (*I*) Quantitation of Ki67-positive cells per crypt, normalized to DAPI-positive cells per crypt in wild type (WT) and *Il10*$^{-/-}$ animals. (*J*) Model of Indole effects during intestinal homeostasis and in response to acute stressors. Commensal-derived or exogenously supplied indoles such as ICA work through IL22 and Type I IFN to control protective responses to acute stressors, or, alternatively, promote healthy aging and limit inflammaging via IL10-mediated effects on goblet cell numbers and intestinal homeostasis. Statistics: Each dot represents an individual mouse. Twenty full crypts counted per section. Data expressed as mean ± SEM. Mann-Whitney tests (*A* and *C*) or Kruskal-Wallis tests with multiple comparisons (*D*, *E*, *G* and *H*) were performed to calculate *P* values.

CHAPTER IV

Effects of Indoles Beyond the Intestine

Abstract

In elderly humans, there is a loss of muscle mass and function that leads to decreased mobility, falls, fractures and even death, and there is currently no effective remedy. Frailty with age, clinically measured by decreases in physical function, is associated with dysbiosis, but it is unclear how changes in the microbiota contributes to health or frailty. We identified indole and its derivatives as molecules secreted by benevolent commensal bacteria that act across diverse phyla (*C. elegans,* Drosophila*,* and mice) to augment health, reduce frailty, and extend healthspan. Indoles derived from commensal bacteria act via Aryl hydrocarbon receptor (Ahr), the anti-inflammatory cytokine IL10, and Type I Interferon (IFN1) signaling to promote healthy intestinal homeostasis, augment intestinal barrier integrity, and limit systemic inflammation. Thus, indoles may limit inflammaging, which can in part be attributed to the leakage of bacterial products across the intestinal barrier that promote systemic inflammation and tissue damage, and ultimately frailty. We have now begun investigating the effects of indoles on systems outside of the intestine. Since previous data has demonstrated that indoles promote motility in aged animals, we decided to investigate effects on muscle tissue. Preliminary data presented here shows that indoles improve muscle regeneration after damage in aged animals and depends on both IL10 and IFN1. We hypothesize that indoles act via Ahr to (i) promote epithelial barrier integrity that limits deleterious systemic inflammatory responses and (ii) promote muscle cell regeneration and stability via IL10 and IFN1. This data shows that indoles may have effects in many body systems and may serve to promote motility in aged animals and limit muscle loss associated age, damage, or disease.

Introduction

Skeletal muscle is a highly regenerative tissue due to the presence of myogenic satellite cells and a high concentration of immune cells within the tissue. The dynamics of the immune response directly influences the dynamics of muscle regeneration from satellite cells (235). In response to damage, a highly structured inflammatory process is initiated, likely through activation of complement by released cytoskeletal proteins or induction of chemokines including CCL2 (236, 237). Within hours of damage, Ly6C+/F4/80- neutrophils begin accumulating in the muscle, followed by phagocytic CD68+ M1 macrophages, peaking between 1 and 2 days post-injury. Though this Th1-dominated phase of the response causes more damage to the muscle, it is essential both to clear cellular debris and to activate satellite cells to proliferate.

After 2 days, M1 macrophages are replaced by nonphagocytic CD206+ and CD163+ M2 macrophages, which peak at day 4 post-injury and remain elevated until the muscle is repaired (235). The transition from Th1 to Th2 response is possibly regulated by the phagocytosis of cellular debris and neutrophils by M1 macrophages. After phagocytosis, the inflammatory macrophages begin expressing transforming growth factor β (TGFβ) and IL10 to drive a switch to M2 macrophages (238). In addition to attenuating further muscle damage, the Th2 dominated response enables satellite cells to enter the early differentiation phase, allowing for muscle growth. Without the Th1-Th2 switch, satellite cells are unable to exit the cell cycle, differentiate, and fuse into muscle fibers (235). Accordingly, mice deficient in IL10 (*Il10*$^{-/-}$) show increased levels of inflammatory cytokines within in the muscle (239), increased muscle fiber damage after injury, and decreased capacity for muscle regeneration and growth (191).

Aging is accompanied by a gradual decline in muscle mass, known as sarcopenia, and a reduction in regenerative potential of muscle in response to injury (240). Inflammaging, an age-

associated increase in levels of pro-inflammatory cytokines and decline in anti-inflammatory cytokines, may be a contributing factor in this change in muscle homeostasis. Elevated levels of inflammatory cytokines, including TNFα and IL6, has been consistently associated with sarcopenia and loss of strength in the elderly (241–243). Additionally, the decline in macrophage phagocytosis observed with age could impact the muscle regenerative process through an inability to clear cellular debris or switch from a Th1 to Th2 dominated immune response (153). Inflammaging is associated with increased frailty (157). Clinical measures of frailty in the elderly focus on physical changes related to muscle function, specifically gait speed (142). Accordingly, we have previously used measurements of motility in diverse model organisms (*C. elegans*, Drosophila, mice) to demonstrate that indoles improve frailty and extend healthspan (166).

Though we have shown indoles improve motility in the aged (166), little is known about the mechanisms by which indoles limit muscle loss during aging or promote recovery from injury. Indoles may limit systemic dissemination of bacterial products across the epithelial barrier, which causes systemic inflammation and accelerated aging (inflammaging), or indoles may protect the muscle itself, or both. In these preliminary studies, we found that indoles use identical signaling pathways to protect muscle as they do to protect the intestine (171, 244); indoles act via pro-inflammatory IFN1 signaling and anti-inflammatory IL-10 signaling to facilitate recovery of damaged muscle.

Materials and Methods

Animals

B6.129P2-*Il10*^{tm1Cgn}/J (*Il10*$^{-/-}$) and C57Bl/6J mice were purchased from Jackson Laboratories. B6(Cg)-*Ifnar1*$^{tm1.2Ees}$/J (*Ifnar1*$^{-/-}$) mice were bred in house. Young mice were used at

3 months. Geriatric mice were aged in house for 2 years. All experiments were performed in accordance with approved guidelines and ethical approval from Emory University's Institutional Animal Care and Use Committee and in compliance with the National Institutes of Health (225).

ICA Administration

ICA was administered at 150mg/kg/day by oral gavage (200ul) beginning 2 days prior to injury and continuing 14 days post-injury.

Muscle Injury for Muscle Regeneration Measurements

Method as described in (245). Mice were anesthetized and given analgesia pre– and post– muscle injury. Anesthetized mice were injected with 1.2% $BaCl_2$ in sterile phosphate-buffered saline (PBS) into tibialis anterior (TA) muscle (20 µl) of one leg. Contralateral leg of the same mouse received sterile PBS injection.

Histology

To analyze muscle regeneration, TA muscles were collected at 14 days post-injury and frozen in Tissue Freezing Media (Triangle Biomedical Research, Cincinnati, OH). Tissue cross sections of 14-µm thickness were collected every 200 µm using a Leica CM1850 cryostat and stained with hematoxylin and eosin. Analyses of regenerated myofiber cross-sectional area were performed using similar anatomical regions of each TA muscle with a total of ~500 myofibers analyzed per genotype for each time point. Myofiber cross-sectional area was measured using ImageJ 1.43u (National Institutes of Health, Bethesda, MD).

Results

ICA Enhances Muscle Regeneration in Geriatric Mice

Recovery of muscle from injury is a key aspect of maintaining motility in geriatric patients. To assess effects of indoles on muscle injury in aged animals, we injected $BaCl_2$ intramuscularly (i.m.) to induce necrosis and harvested tissue at 14 days post-injury (DPI). H&E staining permitted measurement of the area of newly regenerated muscle fibers, identified by centrally localized nuclei. ICA treatment of aged animals (2 years old) resulted in a statistically significant increase in the proportion of muscle fibers with a larger area, indicating that ICA promoted satellite cell proliferation or fiber maturation, or both (Figure 4.1).

Effects of ICA on Muscle Regeneration Depend on IL10 Signaling

We have previously shown that effects of ICA on intestinal homeostasis depend on IL10 (244), and IL10 is essential for initiating the Th2 transition that allows for muscle differentiation and growth during muscle regeneration. To determine whether IL10 mediates the effects of ICA on increasing the area of regenerating muscle fibers in aged mice, we used *Il10*$^{-/-}$ mice in the $BaCl_2$ injury model and again measured the area of centrally nucleated fibers by H&E staining. Indole-mediated increases in fiber size following $BaCl_2$ injury were abrogated in IL10$^{-/-}$ animals at 14 DPI (Figure 4.2 A-B).

Mice Deficient in IFN1 Signaling Show Worse Regeneration in Response to ICA

We have also previously shown that indoles promote barrier integrity by activating stem cells at the base of intestinal crypts via Type I IFN (IFN1) signaling. Since muscle regeneration depends on the activation of satellite cells to proliferate, we used mice deficient in the IFN1

signaling receptor (*Ifnar*$^{-/-}$) in the $BaCl_2$ injury model. Although fibers regenerated normally in the absence of ICA in *Ifnar*$^{-/-}$ animals, ICA slightly reduced fiber size in these mice (Figure 4.2 C-D).

Discussion

Advances in healthcare have contributed to a significant increase in life expectancy, especially in developed countries where geriatric populations are predicted to expand by as much as 350-fold over the next 40 years, accompanied by massive and unsustainable increases in healthcare expenditures (246). Though aging is inevitable, the development of frailty with age may be modifiable. There is a need to develop means to extend healthspan, which is defined as the length of time that an individual remains free of age-related infirmities (166, 247). At the cellular level, healthspan corresponds to the capacity of tissues to resist stressors or to rapidly regenerate via stem cells. At the organismal level, healthspan corresponds to increased periods of motility. Our prior studies demonstrate that indoles derived from the commensal microbiota work through Ahr to improve motility in aged animals, therefore prolonging healthspan (166). Here, we show that providing ICA to aged mice (2 years old) enhances the ability to regenerate muscle in response to injury (Figure 4.1).

We previously demonstrated that the effects of ICA in improving intestinal homeostasis of aging mice was dependent on IL10 (244). Through promoting intestinal barrier integrity to limit access of antigens that cause systemic inflammation and through dampening inflammation, increasing IL10 in the aged may ameliorate inflammaging, which is linked with development of frailty and declines in muscle function. Additionally, ICA induced IL10, likely from macrophages, may facilitate the M1-M2 transition during muscle regeneration and thereby promote satellite cell

differentiation, fusion, and maturation. This enhancement effect is blocked in *Il10*$^{-/-}$ animals, where only baseline differentiation occurs (Figure 4.2 A-B).

We have additionally shown that indoles improve intestinal regeneration in response to damage via IFN1 signaling (171). Effects of IFN1 signaling in muscle are not currently known. However, in other tissues damage signals and toll-like receptors (TLRs) act together with cytoplasmic DNA or RNA sensors STING and RIGi to induce IFN1 signaling and amplify recruitment of monocyte/M1 macrophages to sites of damage (184). Additionally, IFN1 have been shown to activate M1 macrophages (248) and are capable of promoting activation of other types of stem cells (249). Through any of these mechanisms, indole induced IFN1 signaling could enhance the initial phase of muscle repair, which includes clearing of cellular debris and activation of satellite cells to proliferate (235).

Baseline regeneration of muscle fibers following injury occurs with or without ICA. However, with damage signals (DAMPS) present, ICA may activate IFN1 signaling, perhaps in the muscle satellite cells or in M1 macrophages, to amplify M1 recruitment and consequently satellite cell (SC) proliferation. We surmise that in *Ifnar*$^{-/-}$ animals, however, such enhanced M1 recruitment does not happen, which does not affect basal fiber regeneration. However, with ICA present, induction of IL10 by ICA in these animals may inappropriately activate the M1-M2 transition in existing macrophages and enhance satellite cell differentiation into muscle fibers. Without also amplifying recruitment, sufficient and timely expansion of satellite cells is precluded, thereby compromising the repair response, which results in smaller fibers (Figure 4.2 C-D). We hypothesize that the combination of IFN1 and IL10 is required to generate larger fibers after ICA treatment. Amplified monocyte/M1 recruitment following damage together with facilitating the

M1 to M2 transition will allow satellite cells to first proliferate and then differentiate, thereby creating a more robust regenerative response.

We hypothesize that indoles enhance recruitment or activation of M1 macrophages via IFN1 signaling, resulting in removal of cellular debris and activation of satellite cells to proliferate, and then stimulate the transition from M1 to M2 macrophages via delayed IL10 induction, resulting in myogenic differentiation, fusion, and growth. Together these mechanisms facilitate muscle regeneration, particularly in the aged where regenerative capacity is impaired (240).

Continuation of this study will determine how indoles alter immune cell infiltration and satellite cell proliferation, differentiation and maturation following damage or during aging. Using IL10 and interferon stimulated gene (ISG)-GFP reporter mice will allow us to determine the timing of the responses following ICA treatment and injury. Further, using *Ahr* $^{-/-}$, *Il10*$^{-/-}$, and *Ifnar* $^{-/-}$ knockout mice in conjunction with injury and aging models will allow us to determine how indoles utilize IL10 and IFN1 signaling to augment satellite cell proliferation and differentiation. We expect that *Ahr* deficiency will also abrogate enhanced regeneration as we have previously demonstrated indole effects on healthspan to be Ahr dependent (166). Immunostaining for M1 and M2 macrophages following ICA treatment and injury in these mice will show us which phase of muscle regeneration is lost due to the genetic deficiency. Additionally, staining for markers differentially expressed in satellite cells during proliferation vs. differentiation in the knockout mice following ICA treatment will show us which factors are required for ICA-mediated impacts on satellite cell dynamics. Finally, we plan to generate macrophage or satellite-cell specific *Ahr*, *Il10* or *Ifnar* knockout mice then perform $BaCl_2$ injury experiments. We anticipate that in order for ICA to enhance regenerative responses following injury, IL10 will be required in macrophages, and Ahr and IFN1 will be required in satellite cells.

The connection between the intestinal microbiota and skeletal muscle, or "gut-muscle" axis, has recently begun to be appreciated (250). Germ-free mice exhibit muscle atrophy (251), and depleting the microbiota with antibiotics results in reduced muscle endurance (252). Studies attempting to identify specific members of the microbiota responsible for promoting muscle health have associated the presence of bacteria from the family *Prevotellaceae* with athletic performance in young people and with muscle strength in the elderly (253–255). Short-chain fatty acids (SCFAs) produced by these bacteria may be beneficial, as administration of SCFA to germ-free mice improved muscle strength (251). Colonization of germ-free mice with the microbiota of non-frail, high-functioning elderly people also improved muscle strength (253), indicating an association of the microbiota with preventing frailty and sarcopenia with age.

The gut-muscle axis posits that dysbiosis results in increased intestinal permeability and circulating bacterial products that increase inflammation. This inflammation can promote muscle atrophy which leads to impaired muscle function and increased frailty (250). In this regard, interventions focused on the microbiota are an attractive option for reducing frailty and thus precluding age related infirmity. Because the elderly represent the fastest growing patient demographic in the US, these therapies would have enormous impact (246). Since dysbiosis can result in decreased indole production by the microbiota (256), and since we have previously demonstrated that indoles regulated intestinal barrier homeostasis during aging , indoles represent a potential link between the microbiota and skeletal muscle. Data provided here suggest indoles may also have therapeutic utility in promoting muscle regeneration in aged patients, particularly after falls. The discovery that indole induced IL10 affects systems outside of the intestine has opened exciting new areas of study for our lab going forward.

Figures

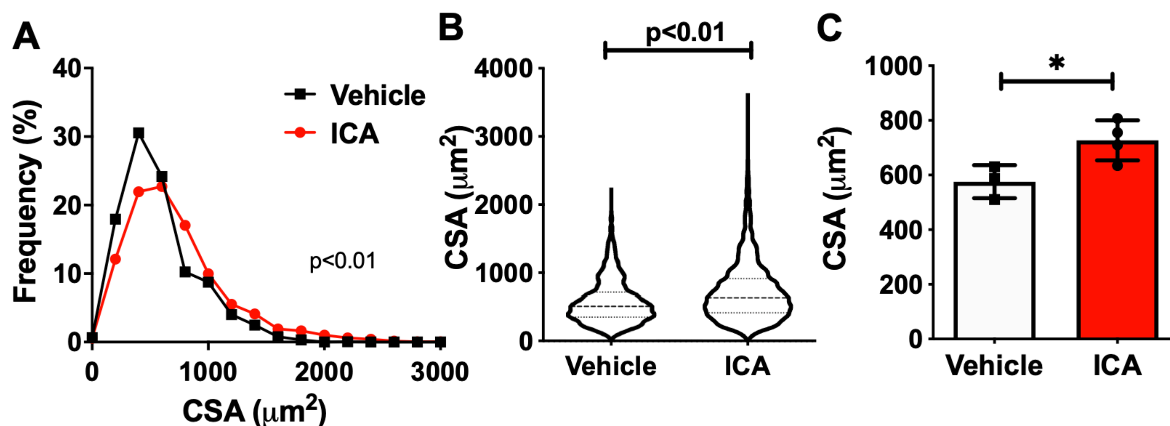

Figure 4.1 ICA enhances muscle regeneration in geriatric mice. Animals were treated with ICA or vehicle for 2 days prior to and during 14-day injury period. (*A*) Frequency histogram of cross-sectional area (CSA) of muscle fibers 14 days post $BaCl_2$ injury. Animals were treated with ICA or vehicle for 2 days prior to injury and throughout the recovery period. Data represent CSA of 1500 fibers from 3 vehicle mice and 1900 fibers from 4 ICA-treated mice. (*B*) Violin plots of CSA data from *A* (Mann Whitney P<0.01). (*C*) Average CSA per mouse from vehicle- (n=3) or ICA-treated (n=4) animals. Data presented as mean +/- SEM. *P<0.05.

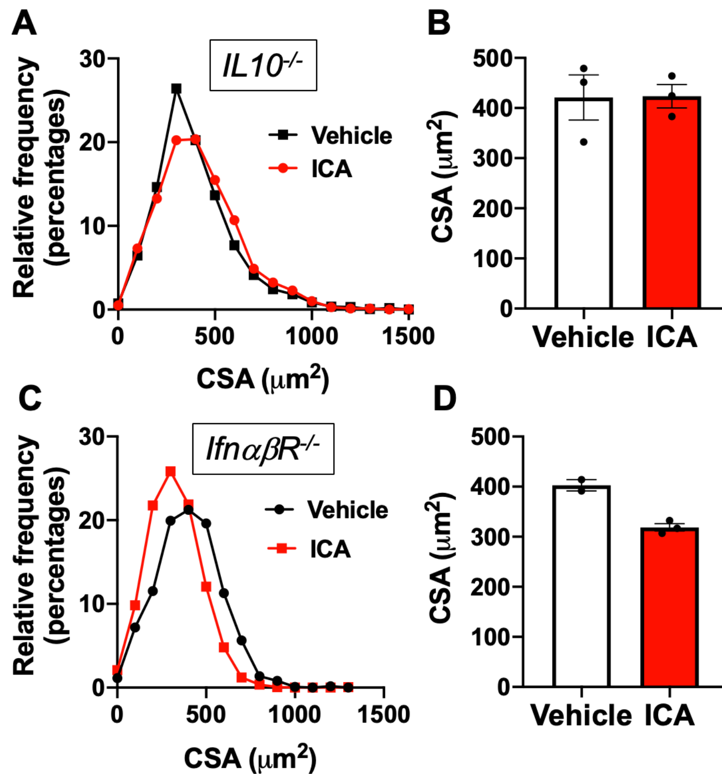

Figure 4.2 Effects of ICA on cross-sectional area after BaCl2 injury depends on IL10 and Type 1 Interferon signaling. Animals were treated with ICA or vehicle for 2 days prior to and during 14-day injury period. (*A*) Frequency histogram of cross-sectional area (CSA) of muscle fibers 14 days post $BaCl_2$ injury in *il10*$^{-/-}$ mice. (*B*) Average CSA per mouse from vehicle (n=3) or ICA-treated (n=3) *il10*$^{-/-}$ mice. (*C*) Frequency histogram of cross-sectional area (CSA) of muscle fibers 14 days post $BaCl_2$ injury in *ifn*αβ*R*$^{-/-}$ mice. (*D*) Average CSA per mouse from vehicle (n=2) or ICA-treated (n=3) *ifn*αβ*R*$^{-/-}$ mice. Data presented as mean +/- SEM.

CHAPTER V

Discussion

One of the most significant advancements modern medicine has seen is the increase in human lifespan, leading to an increased proportion of people age 65 and older in our populations (211). However, the years of added life are often spent in a state of extended frailty, or increased susceptibility to stressors and age-related disease (ARD), rather than extended health. There is now a need to focus on increasing healthspan, which is years of life free from age-related infirmities, rather than lifespan (247). In this regard, we have identified indole and its derivate indole-3-carboxaldehyde (ICA) as molecules derived from commensal bacteria that act across diverse phyla (*C. elegans*, Drosophila, and mice) to reduce frailty and allow animals to live healthier for longer (166).

Inflammaging is the low-level, chronic inflammation that develops over a lifetime of exposure to inflammatory stressors and is associated with increased frailty (96). With age, dysbiosis and a decline in intestinal barrier integrity contribute to increased inflammation originating at the intestinal barrier (211). Indoles increase integrity of the intestinal epithelial barrier and thereby mitigate damage in response to various stressors, including environmental stressors (e.g. radiation), inflammatory immune responses, (171) and pathogens (170) (Figure 1). The objective of the work set forth in this dissertation was to determine how indoles orchestrate stress tolerance and repair responses in the intestinal tract and thereby mitigate inflammatory responses and extend healthspan. While aging is inevitable, the degree to which one experiences frailty or health with age may be modifiable. The concepts and mechanisms developed here are also broadly applicable to diverse stressors that result from dysbiosis or increased barrier permeability.

The Role of Goblet Cells in Health

The capacity of the intestine to continuously regenerate via proliferation and differentiation of crypt stem cells is a vital component of barrier function (11). Of particular interest to intestinal barrier function is the differentiation of goblet cells that produce the mucus layers that limit contact of intestinal bacteria and antigens to the underlying epithelial layer, thus protecting from inflammatory responses (41). To determine the role indole supplementation plays in homeostatic function of the intestinal barrier system, we assessed proliferation and differentiation in the colon with ICA over a period of two-weeks. Here, we have shown that indoles increase the proportion of goblet cells in colonic crypts and promote turnover of cells in the crypts during homeostatic conditions (Figure 3.1). Moreover, using knockout mice, we were able to demonstrate that the effects of indole on goblet cell differentiation and stem cell proliferation are dependent on AHR and IL10, but not on stress-induced IFN1 or IL22 responses. In the knockout mice used, we still observed the same baseline goblet cell numbers as in their untreated wild-type counterparts. Additionally, germ free mice still have goblet cells, but rather lack mucus production (257). This indicates that while there are intrinsic mechanisms that control differentiation from intestinal stem cells, this process is modifiable by environmental sensing of molecules produced by the microbiota.

Most importantly, we demonstrated that aged animals with low health scores (166) also have fewer goblet cells in their colons, an effect of age reversed by colonization with indole-producing K12 bacteria or administration of ICA (Figure 3.2). It is unlikely that goblet cells alone contribute to increased healthspan with indoles especially since we did not see improved thickness of the inner mucus layer. It still remains possible that changes in the quality of the inner mucus layer could improve the ability of the intestinal barrier to exclude potentially inflammatory

antigens. However, our observation that ICA acts via IL10 (Fig. 3.4) may provide an important link between indoles and inflammaging. IL10 is well recognized for its anti-inflammatory activity and decreases with age (148). Moreover, mice deficient in *Il10* exhibit increased inflammation throughout their lives and are increasingly susceptible to inflammatory conditions as they age, including colitis (74, 159). Given increased baseline inflammation, *Il10*$^{-/-}$ mice exhibit increased signs of frailty, including decreased mobility and early mortality (232, 233). Accordingly, in a study of frail geriatric humans, amounts of circulating bacterial muramyl dipeptide was inversely correlated with IL10 levels (162). IL10 induced by indoles may certainly protect against the onset of frailty during aging by limiting decline in goblet cells, and thus barrier function, that would permit translocation of bacterial products from the lumen into the lamina propria. In this regard, have shown previously that ICA treatment of allo-BMT recipients reduced colonic inflammation, IL6, and bacterial leakage into the mesenteric lymph nodes (171). Additionally, IL10 induced by indole treatment may function systemically to limit the extent of inflammatory responses, which is especially important during aging (159).

It has been proposed that with age, the intestinal barrier becomes more permeable, and leakage of bacterial antigens from the lumen into the body cavity precipitates the systemic inflammatory response known as inflammaging (3, 4, 31). However, evidence in support of this theory inflammaging has remained somewhat limited due to the complexity of a multitude of factors involved in aging. By demonstrating a decline in goblet cells with age, we have provided a potential link between intestinal barrier function and inflammaging. Diminishing numbers of goblet cells and thinning of the mucus layer during aging could lead to altered host-microbe relationships and increased exposure to circulating luminal antigens, and, consequently, increased inflammation (229, 230). Additionally, reduced mucus production could contribute to

constipation, a common complaint among the elderly (231), and permit abrasion by feces, leading to a damaged epithelial barrier and inflammation.

Because dysbiosis has been associated with deleterious changes with age (212), it is possible that decreased indole production by the intestinal microbiota could lead to changes in barrier permeability and/or increased inflammation. Measuring levels of indole or its derivatives in the colons of geriatric human subjects and correlating with measures of frailty may provide associations that could be useful as a predictive metric of health. In prospective bone marrow transplantees, low levels of urinary indoxyl-3-sulfate (I3S), the liver metabolite of indole, predicts susceptibility to intestinal GvHD and mortality. Higher levels of I3S is associated with a microbiota composition that limits intestinal inflammation (234, 256). Interestingly, low goblet cell numbers is also associated with more severe GvHD (258). We have previously demonstrated that indoles protect against radiation-induced intestinal inflammation and reduce GvHD pathology (171). Therefore, indoles may protect against GvHD by increasing intestinal goblet cell numbers by improving intestinal barrier integrity and reducing inflammatory responses to translocated bacteria (259).

Goblet cells have long been an underappreciated cell type (260). Over the past few years, several novel functions for goblet cells have been discovered including the ability to sense microbes and secrete mucus in response to inflammasome activation (52, 261) and to deliver antigen to intestinal dendritic cells (262, 263). Additionally, the role of the mucus produced by intestinal goblet cells in selecting for which microbes can reside within the intestinal tract through binding sugar residues and IgA (54) is another rich area for exploration. Future studies focusing on the role on goblet cells and mucus play in health and the consequences of their decline with age will certainly be fruitful. Further delineation of the signaling pathways by which indoles modulate

goblet cell differentiation will help illuminate potential targets for therapeutics focusing on improving the intestinal barrier and promoting a healthy microbiota.

Significance of Understanding Inflammaging

The concept of inflammaging was first described in 2000 by Franceschi et al. as chronic low-level inflammation resulting from continuous antigen exposure over the course of life that leads to a decreased capacity to cope with stressors with age (143). As detailed in Chapter I of this dissertation, additional hallmarks of the aging immune system include decreased anti-inflammatory IL10 and Treg responses, inefficient pathogen responses from both the innate and adaptive immune system, and a predominance of memory B and T cells at the expense of naïve cells. This immune dysfunction leads to increased susceptibility to ARD and infections, increased immunopathology after immune system activation, and ultimately results in frailty (96, 153, 154).

The importance of understanding and addressing inflammaging has been brought to light by the ongoing coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 very disproportionately affects older people, especially those with underlying conditions typically considered ARD (264), including diabetes mellitus (265, 266) and cardiovascular disease (267). In the United States, an overwhelming majority of deaths has occurred in people over the age of 65, with people over the age of 85 representing the highest risk-factor (268). However, since COVID-19 also disproportionally affects young people with ARD, it seems that biological age is a major predictor of COVID-19 mortality (269). Biological age, rather than chronological age, more accurately captures the risk of developing ARD (138) and potentially susceptibility to COVID-19 (270).

A non-human primate (NHP) study demonstrated that the initial response to SARS-CoV-2 is characterized by an influx of myeloid cells, including inflammatory monocytes, neutrophils, and plasmacytoid dendritic cells, into the lungs. The resulting pro-inflammatory and type 1 interferon (IFN1) responses lead to clearance of the majority of the virus (271). Similarly, in people who experience mild COVID-19, it is thought that recruited immune cells efficiently clear the virus and the inflammatory response is then terminated. However, when a dysfunctional immune response occurs, as happens with inflammaging and immunosenescence, there is a hyperinflammatory response that can progress to septic shock and/or multiple organ failure (272, 273). Elevated proinflammatory cytokines, including TNFα and IL6, have been correlated with more severe disease progression (273, 274) as has increased presence of CD14+CD16+ inflammatory monocytes with high IL6 expression in the lung (275, 276). Severe disease has also been associated with reduced cytotoxic T lymphocytes that are required to control viral infection (275), consistent with observations of immunosenescence in aged people (153). An older study of cynomolgus macaques infected with SARS-CoV HKU39849 demonstrated that pathology was worse in older animals due to a more robust inflammatory response compared to young animals despite similar viral loads. Intriguingly for us, an IFN1 response that was anti-inflammatory in young animals was impaired in old animals (277).

Since the COVID-19 pandemic is an ongoing situation, there is still a great deal of research needed to fully understand the pathogenesis of this disease. However, hyperinflammation and immune dysfunction are consistently reported to be associated with worse disease progression, indicating the need for a way to address these responses. As indoles induce anti-inflammatory IL10 responses (244) and reparative IFN1 responses, critically while maintaining T cell responses (171), they may represent potential therapeutics for COVID-19 or other hyperinflammatory ARD.

Additionally, levels of urinary I3S in COVID-19 patients may correlate with disease severity and may be able to inform treatment course.

Future Directions

The significance of this data presented in this dissertation has been further strengthened by our ability to reproduce it in independent model systems and extend the results to new areas of study. We have been able to corroborate the result of indole and IL10 dependent goblet cell development in a human-derived induced pluripotent stem cell (iPSC) intestinal organoid model. Organoids treated with indole showed only a small increase in the number of Muc2+ goblet cells, while direct treatment with IL10 resulted in a prominent expansion of goblet cells (Figure 5). Further use of this model system will allow us determine whether indole itself can induce goblet cell differentiation or if epithelial cells respond to indole treatment by producing IL10. Additional use of mouse models will allow us to understand what cell types are responding to indole and IL10 and therefore which cells should be targeted by therapeutics.

The work in this dissertation has set also the groundwork for investigating the role of microbiota-derived indole induced IL10 in other organ systems outside of the intestine. As discussed in Chapter IV of this dissertation, one area currently under investigation in our lab is age-related sarcopenia. A major determinant of frailty is decreased muscle strength and function which manifests in physical traits such as low activity levels and slow gait speeds (141, 242). *Il10*$^{-/-}$ mice, used as a model of frailty, display muscle weakness early in their lives (278). We have so far shown that indoles improve muscle regeneration in old mice following injury in a manner dependent on both IL10 and IFN1. Future studies will elucidate whether these phenotypes are

mediated by ICA effects on the intestinal barrier, by global IL10 expression, or on dynamics of the transition from Th1 to Th2 driven immune responses during muscle regeneration (235).

Conclusion

This dissertation contributes to our understanding of how the commensal microbiota is a determinant of health status, especially during aging. Activation of IL10 by indoles derived from the commensal microbiota likely influences multiple aspects of the intestinal barrier under homeostatic conditions. Indoles initiate protective reorganization of the epithelial barrier via upregulation of goblet cell differentiation and other protective mechanisms, such as increasing transepithelial resistance (90, 171) and regulation expression of the IL10 receptor (IL10R) on intestinal epithelial cells (90), *and* simultaneously limit systemic inflammatory responses to bacterial antigens through Ahr-dependent IL10 induction. Together, these observations suggest that indoles are multifaceted microbiota-derived factors that can protect the epithelial barrier, especially during aging, and limit inflammation associated with transit of bacterial products. As a result, these findings will inform the design of therapeutics that limit detrimental changes associated with inflammaging or other inflammatory diseases and promote healthspan, the capacity to live better for longer.

Figures

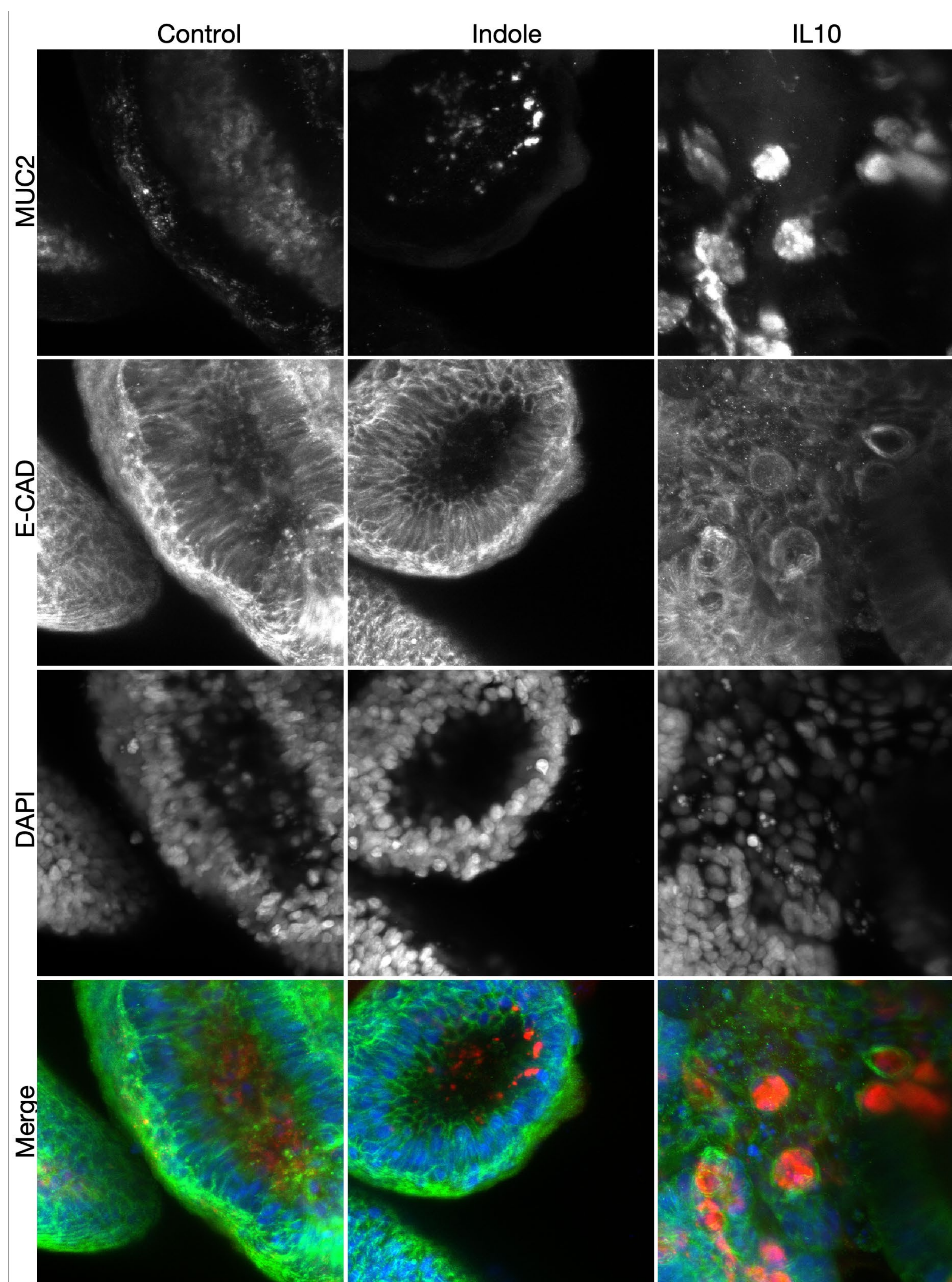

Figure 5. Indole and IL10 induce goblet cell differentiation in a human-derived iPSC intestinal organoid model. Human-derived pluripotent stem cells were induced to develop

intestinal organoids. Organoids were treated with indole (center column) or IL-10 (right column) for 1 week. Muc2 (red) indicates goblet cells. E-CAD (green) marks epithelial cell boundaries. DAPI (blue) stains nuclei.

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