THE MICROBIALLY DERIVED METABOLITE INDOLE ATTENUATES OBESITY ASSOCIATED INFLAMMATORY PROCESSES IN ADIPOCYTES AND MACROPHAGES

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2016

Major Subject: Chemical Engineering

ABSTRACT

The human gastrointestinal (GI) tract is colonized by ~10^{14} bacteria belonging to ~1,000 species that are collectively termed the intestinal microbiota. Recent studies show that the intestinal microbiota impacts a wide range of functions in the GI tract including development of the immune system, defense against pathogens, and inflammation. Indole is derived from dietary tryptophan from microbial enzymatic activity. Previous work from our laboratory identified indole attenuates indicators of inflammation, increases tight junction resistance and increases colonization resistance in intestinal epithelial cells. This work identifies the effects of indole extend to both adipocytes and macrophages beyond intestinal epithelial cells. Indole attenuates LPS-mediated induction of TNF-α production in RAW264.7 murine macrophages. Additionally, indole also attenuates LPS-mediated MCP-1 expression in 3T3-L1 adipocytes. The inhibition of MCP-1 expression in adipocytes was mirrored by a significant attenuation of bone marrow derived macrophage migration toward MCP-1 and adipocyte conditioned media. Indole correspondingly inhibits macrophage stimulated media induction of MCP-1 in adipocytes. Therefore indole interferes with paracrine signaling between macrophages and adipocytes. Indole is also exhibits agonist and antagonist activity for the AhR. However, the indole-mediated decreases in macrophage chemotaxis towards MCP-1 and in LPS induction of TNF-α production in BMDMs are independent of the AhR. Further, utilizing a novel indole-protein conjugate, we show the effects of indole do not require uptake of indole into the cytoplasm. In addition, this work also demonstrates that indole

may signal through the mTOR pathway in macrophages. These studies suggest indole exerts effects on inflammatory processes by altering the mTOR signaling pathway and suggest an alternative mechanism of action to the recently identified effects of indole on the AhR pathway.

DEDICATION

Love. You can learn all the math in the 'verse, but you take a boat in the air you don't love, she'll shake you off just as sure as the turning of worlds. Love keeps her in the air when she oughta fall down, tells you she's hurting 'fore she keens. Makes her a home [1].

ACKNOWLEDGEMENTS

I would like to thank the selfless dedication of the Jayaraman lab members, past and present, as well as the Alaniz Lab and Lee Lab members. In particular, Daniel Howsmon and Lakshmi Nathan, two incredibly talented undergraduate researchers made critical contributions to this work. I would also like to thank our collaborators Dr. Steve Safe, Dr. Robert Chapkin and Dr. Kyongbum Lee. Finally, I would like to thank my committee for providing their insightful critique of this work.

CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by a dissertation committee consisting of Professor Arul Jayaraman, advisor, and Professors Katy Kao, Hung-Jen Wu of the Department of Chemical Engineering and Professors Julian Leibowitz and Robert C. Alaniz of the Department of Microbial Pathogenesis and Immunology. All work for the dissertation was completed independently by the student.

Graduate study was supported by a research assistantship from the Department of Chemical Engineering. This work was supported by a grant from the NSF-CBET 1264526 to Arul Jayaraman of the Department of Chemical Engineering.

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1. INTRODUCTION

The human gastrointestinal (GI) tract is colonized by ~10^{14} microorganisms belonging to ~1,000 species that are collectively termed the intestinal microbiota [2]. Recent studies show that the intestinal microbiota impacts a wide range of functions in the GI tract including development of the immune system [3], defense against pathogens [4], and inflammation [4]. Beyond the GI tract, alterations in the microbiota have been correlated with the development of obesity and insulin resistance [5-7]. The microbiota impacts bile acid production and hormone regulation in the liver [8], and dysbiosis of the microbial community has been linked to the development and progression of non-alcoholic fatty liver disease (NALFD) [9].The gut also influences the function of other organ systems as gut-brain [10], gut-lung [11], and gut-liver [12] links have also been identified.

Obesity, defined as a significant expansion of the adipose tissue, is associated with several severe diseases including type 2 diabetes, coronary heart disease and cancer. Obesity related diseases such as insulin resistance and diabetes are a serious problem in the modern developed world as the obese population continues to expand [13]. Diabetes related health cost in 2012 was $245 billion [13], a significant increase from previous annual diabetes related costs in 2007 of $113 billion (2007 dollars) [14]. Despite multiple FDA approved treatment options, there is no clear optimal therapeutic treatment option due to uncertainties in treatment adverse effects and overall benefits of treatment

[15]. Understanding the fundamental biological events in the development of chronic obesity related inflammation leading to insulin resistance will allow better treatment and care to be established to slow or even halt development of the disease.

The microbiota has been recently identified as a factor that influences the onset and progression of obesity-induced inflammation to insulin resistance and diabetes [16, 17]. Obesity causes a pronounced shift in the composition of the microbiota (i.e., dysbiosis) and the dysbiotic microbiota in obesity contributes to the progressive deterioration of host insulin sensitivity to type 2 diabetes [18-20]. The dysbiotic microbiota has an increased capacity for energy harvest and contributes to alterations in host metabolism, inflammatory state, and epithelial barrier function [16, 20, 21]. High fat diet induced obesity (DIO) [22] has become a standard murine model for studying obesity induced insulin resistance [23]. Gnotobiotic mice studies (i.e., mice raised free of microbial organisms and colonized with specific microbial populations or species) have played a pivotal role in understanding the contribution of the microbiota to obesity and other diseases. Gnotobiotic mice colonized with a human-derived microbiota and raised on a plant rich diet rich in polysaccharides, then shifted from a plant rich diet to a high fat diet display an altered gut microbiota within a single day [24]. Other gut-organ axes correlate have been identified and alterations in the intestinal microbiota contribute to the development of host diseases including obesity [25], diabetes [26], cancer [27], and asthma [28]. These studies affirm the importance of the microbiota in whole body homeostasis and that microbiota dysbiosis significantly impacts the onset and

progression of several diseases. It is therefore of great interest to understand mechanistically how the microbiome contributes to the progression of disease and if the microbiome itself can be modulated to dampen or prevent development of these diseases.

Several models have been proposed for describing LPS-mediated inflammation in obesity. Cani et al. proposed that a leaky gut epithelial barrier in obese subjects allows increased levels of bacterial lipopolysaccharide (LPS) to enter into circulation and initiate adipose tissue inflammation [19]. In this chronic model of LPS induced inflammation, LPS acts as an initial inflammatory stimulus causing adipocytes and resident adipose tissue macrophages to produce pro-inflammatory cytokines and chemokines in response to the elevated levels of endotoxin [20]. Adipocyte and macrophage derived cytokines and chemokines such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and adiponectin (ACDC) and monocyte chemoattractant protein 1 (MCP-1) have direct causative roles in the development of persistent chronic inflammation and insulin resistance [18, 29-31]. These cytokines persist at elevated levels in circulation in the obese state, leading to chronic activation of the adipose tissue.

In addition to an increase in cytokine production by adipocytes and resident tissue macrophages, obesity induces a phenotypic switch in macrophage populations in the adipose tissue [32]. The macrophage has been traditionally described as a phagocytic cell responsible for performing immune surveillance and homeostatic maintenance

including the removal of apoptotic and senescent cells[32, 33]. Due to the differing microenvironments local macrophages experience in various tissues in the body, resident tissue macrophages have equally broad roles in maintenance of specific tissue types [34]. The phenotypic activation state, or polarization state, of macrophages broadly describes the roles of macrophage populations in tissue remodeling, inflammation and obesity [20, 32, 35]. Macrophage polarization is classically defined by three factors which characterize how the macrophage functions in the context of the host tissue; the metabolic macrophage state, the expression of cell surface receptors and the production of functional cytokines [36-39]. The polarization states of the macrophage can classically be defined as either M1 inflammatory macrophages which secrete inflammatory cytokines (e.g. IL-6, TNF-α) in response to a stimulus or M2 regulatory macrophages which promote tissue maintenance and repair [37]. The progression of obesity induced inflammation involves significant accumulation of M1-like inflammatory macrophages to the adipose tissue into localized regions called crown like structures [40]. Importantly, these M1 and M2 states are not rigidly fixed, and in fact macrophages can exist in many different states between M1 and M2 [37], and the functional state of macrophages is conveniently described by these relative terms.

Obesity is a metabolic disorder as well as an immunologic disorder as the dysregulation of adipose tissue macrophages has a causative role in the progression of insulin resistance and type 2 diabetes [41, 42]. One cellular signaling pathway at a crossroads of host metabolism and the immune system is the mechanistic target of rapamycin (mTOR)

pathway [43]. mTOR functions as a serine threonine protein kinase in the phosphoinositide 3-kinase (PI3K)-related kinase family and resides in cells as part of two distinct complexes mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [44, 45]. The classic immune modulator rapamycin, from which mTOR is named, inhibits mTORC1 by directly binding FK506 binding protein (FKBP12) and forming a complex with FKBP12 [46]. This complex then binds to and inhibits mTORC1 activity [47].

The most well characterized roles of mTOR are its effects on protein synthesis through the activation of protein translation [48]. mTOR also serves as a regulator of the synthesis of lipids through its regulation of sterol regulatory element-binding protein 1/2 (SREBP1/2) [49]. Further, mTOR is involved in the regulation of cell metabolism through activation of hypoxia inducible factor 1 (HIF1α) [50], and sensing of amino acid availability through the solute carrier family 1 member 5 (SLC1A5) solute carrier family solute carrier family 7 member 5 (SLC7A5) pathway [51]. Aside from roles in maintaining cellular processes during variable nutrient availability and aiding in defense against pathogens, the mTOR pathway has been implicated in an increasing number of pathological conditions including obesity and type 2 diabetes, cancer and neurodegeneration [52-54]. mTOR regulation has a central role in macrophage polarization through the tuberous sclerosis proteins (TSC) 1/2 complex which negatively regulates mTOR [55]. The involvement of mTOR in the development of macrophages is of great interest as it links a metabolic regulation pathway to the regulation of the innate

immune system. Endogenous molecules identified to modulate mTOR signaling would represent an interesting class of metabolic and immune regulators.

Studies have shown that physical interactions between GI tract bacteria and host cells, such as paneth cells directly sensing bacteria through toll like receptor 4 (TLR-4), are important for maintaining intestinal epithelial homeostasis [56, 57]. However, the mechanism(s) involved in the microbiota interactions in distal host sites (i.e. adipose tissue) are not likely due to direct physical contact between the bacteria and the host cells, as bacteria are generally not found in circulation except during sepsis [58]. Instead, metabolic products of the microbiota (microbiota metabolites) are thought to function as effector molecules at both local and distal sites [59]. Consistent with this notion, microbiota-derived metabolites such as indoxyl sulfate have been detected in circulation of conventional but not germ-free mice [60, 61].

Short chain fatty acids (SCFAs) such as butyrate are well studied microbiota derived metabolites which are used as energy sources by colonocytes [62] and also attenuate inflammation in these cells [63]. Recently, molecules derived from amino acids such as tryptophan have also been shown to elicit a responses both *in vitro* [64] and *in vivo* [65]. Work from our lab demonstrated that indole, which is produced in bacteria from L-tryptophan via the tryptophanase enzyme TnaA [66], attenuated TNF-α-mediated activation of NF-KB and expression of the proinflammatory chemokine interleukin 8 (IL-8) in HCT-8 intestinal epithelial cells [65]. Shimada et al., [64] demonstrated that

indole increased the expression of genes that contribute to tight junction and intestinal barrier function in a murine model and further that indole attenuated indicators of inflammation in a murine model of DSS colitis. Zelante et al. demonstrated a different tryptophan-derived metabolite (indole-3-aldehyde) promoted the transcription of IL-22 to modulate antifungal resistance and mucosal protection [67]. The microbiota also affect host amino acid metabolism as a recent report identified spore forming bacteria produce soluble factors which positively regulate host serotonin biosynthesis [68]. However, to date there are no reports of microbial derived metabolites capable of attenuating the inflammatory processes which cause insulin resistance.

Metabolic products of the microbiota represent a source of bioactive compounds distinct from traditional bacterial cell membrane components such as LPS or structural features such as flagella which activate the immune response through pattern recognition receptors (PRR). Microbial derived metabolites activate a variety of cell surface and intracellular host receptors. The free fatty acid (FFA) receptors including G-protein coupled receptors GPR40, GPR41, GPR43, GPR84 and GPR120 recognize nonesterified fatty acids of varying sizes [69]. Recent work has shown these fatty acids including the SCFA butyrate have an essential role in activating regulatory colonic T-cell populations [70]. Aryl hydrocarbon receptor (AhR) activation has been shown to be altered by several amino acid derived metabolites including indole and other indole-like molecules [61, 71, 72]. Jin et al. show that tryptophan-derived microbiota metabolites are ligands for the AhR in CaCo-2 human colon cancer cells and MDA-MB-468 and MDA-MB-231

breast cancer cells. Specifically, indole increased expression of the prototypical AhR target gene cytochrome P4501A1 (CYP1A1) in a concentration-dependent manner and also antagonized 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) mediated induction of CYP1A1 (14). AhR function is increasingly being appreciated as central to immune cell development and function, particularly in the context of endogenous ligands such as tryptophan metabolites.

The hypothesis underlying this work is that indole attenuates chronic low grade inflammation by reducing the inflammatory response to LPS in adipocytes and macrophages. This hypothesis is based on the recent observation that indole attenuated cytokine activation of NF-κB [65]. We therefore hypothesize as an extension of this work that indole would also attenuates chronic low grade inflammation caused by elevated serum LPS levels. The focus of this work is elucidating the effects of indole on inflammation in macrophages and adipocytes caused by elevated LPS and the underlying mechanisms using *in vitro* models. The above hypothesis will be tested via three specific aims.

Aim1: Determine the effect of indole on the adipocyte and macrophage inflammatory response to LPS. Experiments in this aim will characterize the inflammatory processes that are modulated by indole in an established *in vitro* model of LPS-induced inflammation. In addition, these experiments will also investigate the effect

of indole on cytokine-mediated paracrine signaling between macrophages and adipocytes

Aim 2: Identify the molecular mechanism underlying the effect of indole on macrophage inflammation. This aim will focus on the receptors (intracellular and cell surface) and signaling pathways that are engaged by indole. Approaches to be adopted include development of an indole-BSA (I3-BSA) conjugate that will localize indole to the cell surface and investigating the alteration of the mTOR signaling pathway that is observed with indole. We will specifically focus on the mTOR pathway given its importance in macrophage polarization, metabolism and inflammation.

Aim 3: Investigate the effect of indole on macrophage phenotypes and function. Experiments proposed in this aim will investigate the effect of indole on macrophage metabolism, cytokine production and polarization in response to endotoxin and cytokine polarization skew signals. These experiments will determine the extent of functional changes indole induces in macrophages.

The proposed studies will provide insights into how the endogenous, microbially produced molecule indole affects inflammatory processes which have a causative role in the progression of obesity induced chronic inflammation and insulin resistance.

2. LITERATURE REVIEW

2.1 The microbiota

In the past decade, studies utilizing next-generation sequencing have significantly expanded our knowledge of the microorganisms in the microbial community as well as the cumulative changes in microbial gene expression both in homeostasis and in disease [73]. While several studies have described alterations in the microbial community composition present in the GI tract under specific conditions, a large fraction of the microbial community has not been identified[74]. Characterizing the microbial community down to the species level as well as culturing individual species or sub-populations is the current major challenge in the study of host-associated microbial communities [75].

In the absence of the microbiota, nearly every aspect of the host's physiology is affected [73]. Studies using a germ-free (GF) mouse model that lacks any microbiota have shown that the intestinal microbiota impacts a wide range of functions in the GI tract including development of the immune system [3], defense against pathogens [4], and inflammation [4]. Even perturbations in the microbiota composition (i.e., dysbiosis) due to certain environmental factors (e.g., high-fat diet, contaminant exposure) [76, 77] or medical interventions (e.g., surgery, antibiotic treatment) [78, 79] have a profound effect on host physiology in the GI tract leading to inflammation, obesity, and infections [77, 80-82]. The adverse effects of on the host are not restricted to the GI tract alone. Beyond the GI

tract, gut-brain [10], gut-lung [11], and gut-liver [12] links have also been identified, with microbiota dysbiosis being correlated to diseases such as autism, asthma and non-alcoholic fatty liver disease, respectively [83].

2.1.1 Microbiota in energy generation

One of the primary roles of the intestinal microbiota is in energy harvest. Given the broad diversity in the intestinal microbiota community, it is not surprising that the microbiota is capable of carrying out a broad range of biotransformation reactions, including those that are not present in the mammalian host such as the production of indole from tryptophan [84, 85]. A critical function of the microbiota is the digestion and fermentation of certain dietary polysaccharides which are indigestible by human enzymes to produce SCFA's [86]. These bacterially produced SCFAs have been shown to exert multiple beneficial effects on mammalian energy metabolism including being directly used as an energy source by colonocytes [86], regulation of cholesterol metabolism [87], and the regulation of glucose metabolism [88]. The microbiota also metabolize and transform complex dietary polyphenols, compounds typically derived from plants such as from teas, fruits and vegetables, contain one aromatic structure and one or more hydroxyl group [84]. Consumption of foods high in polyphenols such as flavonoids, stilbenes and benzoic acids has been correlated to reduced risk of cardiovascular disease and cancer [89, 90]. However, the metabolic potential of the microbiota is enormous in comparison to the host as the genetic content of the

microbiota is approximately 150-fold larger than the ~20,000 genes encoded by the host [91]. Unsurprisingly, the field has only just begun to identify functional metabolic products of the microbiota and the roles of these compounds have in the host [92].

2.1.2 Role of the microbiota in the development of the immune system

Despite the symbiotic nature of the microbiota-host interaction in the GI tract, the sheer number of microbes in close association with the host poses an immense immunological threat. However, despite the potential threat, the microbiota contributes to the development and training of the immune system both directly in the gut and indirectly through metabolic products produced by the microbiota and taken up into circulation by the host. The microbiota is directly sensed by intestinal dendritic cells during antigen sampling and handling which trains the immune system to specifically distinguish the commensal microbiota from invading pathogens [57, 93]. [3, 94]. Indirectly SCFA's produced by the microbiota promote effector and regulatory T cells through suppression of histone deacetylases [94]. In the small intestine, GF mice do not develop isolated lymphoid follicles and these mice are also deficient in secretory IgA and CD8αβ intraepithelial lymphocytes [95]. Additionally, colonization of mice by a defined mixture of bacteria belonging to the genus *Clostridium* promoted anti-inflammatory regulatory T-cell (Treg) accumulation [96]. Oral inoculation of this *Clostridium* cocktail during early life of conventionally raised mice also conferred resistance to the development of

colitis in a dextran sodium sulfate (DSS) induced colitis model. The microbiota therefore has several distinct and critical roles in the development of the immune system.

2.1.3 Microbiota in prevention of pathogen infection

The interplay between the microbiota and the host defense against extends beyond simply training the host immune system. Through multiple mechanisms including competitive metabolic interactions, direct action against pathogens and the previously described induction of the host immune response, the microbiota protect the host against foreign pathogen invasion [4]. Intestinal microbiota dysbiosis due to antibiotic administration has been shown to be an important step in the onset of *Clostridium difficile* infections [97, 98]. In fact, several experimental mouse models for studying pathogen colonization and infection (e.g. vancomycin-resistant *Enterococcus*) utilize disruption of the intestinal microbiota with specific antibiotics to facilitate infection [99]. Antibiotic resistant pathogens such as the *Enterobacteriaceae* species *Pseudomonas aeruginosa* and *Acinetobacter baumannii* from the G.I. reservoir emerge after antibiotic treatment regimens destroy competing antibiotic sensitive commensals [100]. Commensal *E. coli* compete for necessary nutrient sources (e.g., the amino acids proline) also utilized by the pathogen enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) and pre-colonization of the intestine with commensal strains of bacteria can prevent pathogen colonization [101, 102]. Commensal bacteria produce bacteriocins, bacterial produced peptides, including the F-type and R-type pyrocins produced by *Pseudomonas*

aeruginosa [103, 104], that kill or inhibit growth of closely related bacteria. Probiotic supernatants produced by commensals such as *Lactobacillus acidophilus* reduce the expression of virulence genes in EHEC involved in pathogen colonization [105]. Work from our lab identified indole as a metabolite produced by the microbiota that attenuates EHEC virulence gene expression, motility, and biofilm formation [106]. Indole treatment decreased surface-associated EHEC expression of genes involved in surface colonization and virulence while the eukaryotic hormones epinephrine and norepinephrine increased expression of these same genes [106]. SCFA's produced by *Bifidobacterium spp.* block the colonization of pathogens by locally acidification of the environment inhabited by pathogenic *E. coli* [107].

2.1.4 The microbiota in disease

Beyond obesity, there are several well established gut microbiota – organ axes in which the presence of the microbiota affects the function of distal organs. Bile acids produced in the liver are further metabolized by the gut microbiota into secondary bile acids and these bile acid pools are co-regulated by the microbiota and the host [108]. The microbiota regulates hepatic bile acid synthesis of muricholic bile acids [8]. The microbiota derived secondary bile acid tauro-conjugated muricholic acids antagonize the farnesoid X receptor (FXR), a nuclear receptor which regulates bile acid synthesis, in the liver [8]. Dysbiosis of the microbial community has been linked to the development and progression of non-alcoholic fatty liver disease (NAFLD), primarily through endogenous

microbial production of ethanol [9, 109]. However, irregular microbial bile acid production has also been shown to alter liver lipid and glucose metabolism and the progression of NAFLD [109, 110]. The microbiota is a key regulator of the host synthesis of the neurotransmitter serotonin and is critical to the normal function of the gut-brain axis [10, 111]. Male germ free mice displayed altered brain concentrations of 5-hydroxy tryptophan, a key neurotransmitter in the regulation of mood, in comparison to mice containing a conventional microbiota [112]. Early life exposure to antibiotics significantly increases the host susceptibility to the development of asthma, consistent with the microbiota being crucial for proper regulation of the gut-lung axis [28]. Dysregulated microbiota and microbiota with a reduced complexity in the phylum *Firmicutes* [113] are essential factors that drive inflammation and the development of inflammatory bowel disorders such as ulcerative colitis and Crohn's disease (CD) [114]. However, microarray data have shown that this is not the case for all members of a phylum. Differences in microbial populations of CD patients down to the species level identified a greater abundance of *Lachnospiraceae* and *Ruminococcaceaea* the family level and a greater abundance *Clostridium difficile, Lactobacillus fermentum* and *Shigella flexneri* at the species level in patients suffering from CD [115]. While the community composition in normal and dysbiotic microbiota is relatively well defined for some disease states at the phylum level with some differences identified to the species level in well studied diseases like CD, the molecules being produced and the mechanism by which these metabolic compounds exert an effect on the host is severely lacking in comparison [116]. These studies highlight the importance of the microbiota in whole

body homeostasis and that microbiota dysbiosis significantly impacts the onset and progression of several diseases.

2.1.5 Microbial derived metabolites

It is clear that the intestinal microbiota exert beneficial effects in and away from the GI tract. These effects are, in part, mediated in part by molecules the community produces. Interactions between GI tract bacteria and host cells, such as *Bacteroides fragilis* derived polysaccharide influencing $CD4^+$ T cell maturation, are important for maintaining intestinal epithelial homeostasis [56, 57]. However, the mechanism(s) involved in the microbiota interactions in other tissues (e.g., adipose tissue, liver) are not likely due to direct physical contact between the bacteria and the host cells, as bacteria are generally not found in circulation except during sepsis [58]. Instead, metabolic products of the microbiota such as indole and SCFA's are thought to function as effector molecules at both local and distant sites [59, 64, 86]. Metabolic products of the microbiota represent a source of bioactive compounds distinct from bacterial cell membrane components such as LPS or structural features such as flagella which activate the immune response through pattern recognition receptors. Several studies have shown that these metabolites impact a wide range of host phenotypes in different cell types and tissues. SCFA's such as butyrate are an essential source of energy for colonocytes, though propionate and acetate can also be utilized by colonocytes [86, 117]. However, the liver utilizes up to 70% of acetate where acetate is used as both an energy source and a precursor for

cholesterol synthesis. In addition, the SCFA propionate lowers cholesterol synthesis rate and both acetate and propionate increase fatty acid oxidation metabolism in multiple tissues. SCFA's also kill pathogenic *Escherichia coli* K12 and *Salmonella* spp. at high concentrations and low pH and butyrate and propionate downregulate *Salmonella* virulence genes [118, 119]. Further, SCFA's such as butyrate have an essential role in activating regulatory colonic T-cell populations as noted above [70]. These observations collectively indicate microbial derived SCFA's have critical roles in host metabolism and immunity beyond simply providing energy to colonocytes.

Recently, microbially produced molecules derived from amino acids such as tryptophan have also been shown to elicit increased intestinal epithelial barrier resistance *in vitro*[65] and *in vivo* [64]. Consistent with this notion, microbiota-derived metabolites such as indoxyl sulfate have been detected in circulation of conventional but not germ-free mice [60, 61]. Indole is in fact an interesting compound as several host, microbiota and diet derived compounds with the indole moiety have been reported to significantly modulate beneficial phenotypes in the host. Work from our lab demonstrated that indole, which is produced in bacteria from L-tryptophan via the tryptophanase enzyme TnaA [66], attenuated TNF-α-mediated activation of NF-KB and expression of the proinflammatory chemokine interleukin 8 (IL-8) in HCT-8 intestinal epithelial cells [65]. Shimada et al. further demonstrated that indole increased the expression of genes that contribute to tight junction and intestinal barrier function in a murine model and further that indole attenuated indicators of inflammation in a murine model of DSS colitis [64].

Zelante et al. demonstrated indole-3-aldehyde promotes the transcription of IL-22 which contributes to antifungal resistance and mucosal protection[67]. Microbiota metabolites of tryptophan modulate astrocyte activity and attenuate inflammation in EAE [120]. However, to date there are no reports of microbial derived metabolites capable of attenuating the inflammatory processes which cause insulin resistance. Spore forming bacteria produce soluble factors which positively regulate host serotonin biosynthesis [68]. AhR activation has been shown to be altered by several amino acid derived metabolites including indole and other indole-like molecules [61, 71, 72].

2.1.6 Mass spectrometry detection of microbiota metabolites

Biological samples of interest (e.g. feces, blood or urine extracts) present a challenge in accurate identification of target compounds. Simultaneous quantification of a large number of metabolites using MS is further complicated due to the large dynamic range of metabolites present in samples. Liquid chromatography – mass spectrometry (LC-MS) methods for analysis of such samples are well established complementary techniques to separate and identify individual or multiple compounds [121]. Separation of individual compounds in LC is achieved by passing the sample in the mobile solvent phase through a chromatography column containing a solid stationary phase, typically made of silica with or without chemical modification. Dissimilar compounds in the sample interact differentially with the stationary phase and pass through the length of the column at different rates, effectively separating the sample into components.

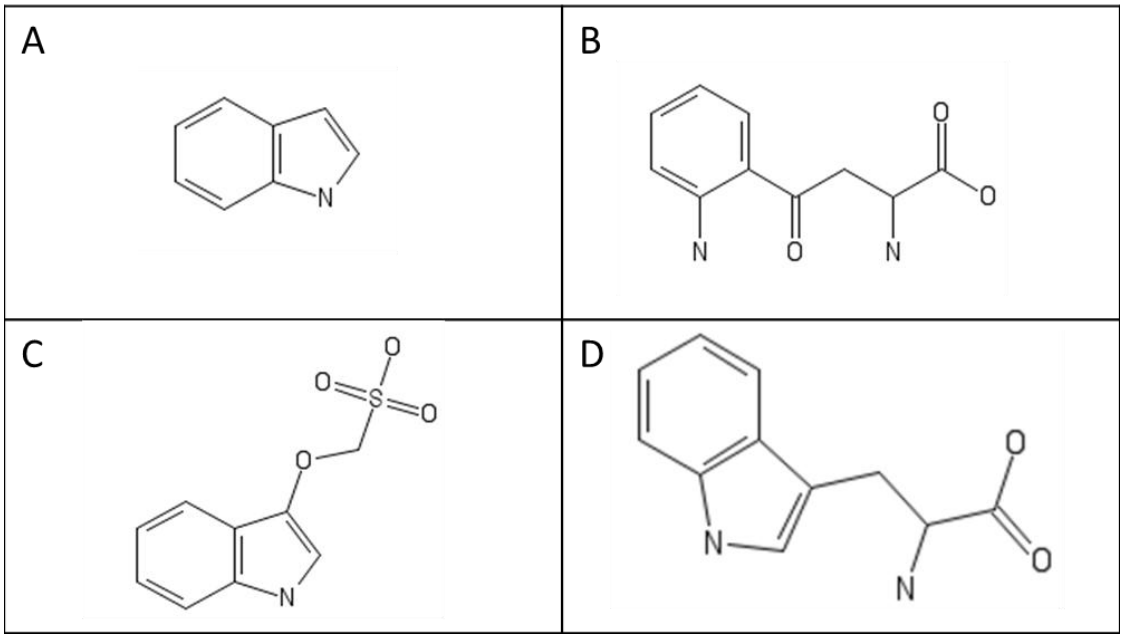


Figure 1. Tryptophan derived metabolites. (A) Indole derived from bacteria conversion of tryptophan via TnaA. (B) Kynurenine derived from host conversion of tryptophan via IDO. (3) Indoxyl sulfate derived from host cytochrome P-450 hydroxylation of indole followed by sulfotransferase of indoxyl. (D) Tryptophan

Several studies have used antibiotic perturbation of the microbiota and analysis of the microbiome in untargeted efforts to identify metabolic products derived from the microbiota. Wikoff et al. utilized untargeted MS analysis to identify ~4000 ions in serum taken from conventional and GF mice and that of these ions, ~10% showed significantly different signal intensity in either conventional or GF serum extracts [60]. The authors further showed that indoxyl sulfate derived from tryptophan by microbial and host metabolism are present in circulation of conventional but not GF mice and that production of indole-3-propionic acid was completely dependent on the presence of the microbiota [122]. Kok et al. used capillary electrophoresis (CE) coupled with MS to characterize the impact of antibiotic treatment on the metabolite profile of rat urine [123]. In this work, antibiotic treatment altered the relative concentration of 17 compounds of the 347 total compounds identified and of these 17 compounds, two were the indole derived compounds indoxyl and indoxyl sulfate. Antunes et al. detected more than 2000 metabolites in murine fecal material and showed a single high dose antibiotic treatment caused significant changes in 90% of these compounds [124].

Single polarity, single ionization untargeted MS experiments exclude large numbers of potential metabolites of interest, though there have been several successful efforts. More often, untargeted liquid chromatography coupled to different ionization techniques produces a larger spectrum of potential metabolites for study [122]. While there is no denying the discovery power of untargeted approaches, untargeted MS based techniques inherently is suboptimal for detection of low concentration or poorly ionized metabolites

due to the optimization of instrument parameters for total ion currents. Unambiguous identification of bacterial metabolites may also present challenges if the sample contains many ion fragments with similar mass signatures. Further, without a predetermined set of targets, an extraction protocol and MS operating parameters cannot be tailored for particular classes of metabolites to improve sensitivity. Ultimately, all workflows contain some degree of sample targeting either in the extraction method, compound separation system or MS analysis though the number of compounds identified in these untargeted studies continues to grow dramatically as instrument power increases. Targeted metabolite identification alleviates several limiting issues of untargeted approaches by tailoring the detection approach to the metabolites of interest. Extraction methods, separation techniques and instrument parameters can be tailored to metabolites of interest. However, targeted approaches present other unique drawbacks including the laborious choice of metabolites which previously had come from biochemical analysis of samples. Computational approaches have contributed to the understanding of living systems and have further aided in the identification of useful bacterial products both for industrial and therapeutic use [125, 126] . Probabilistic approaches such as ProbPath have been developed for targeted metabolic analysis to rapidly identify metabolites predicted to be produced, either solely by the microbiota as in the case of indole or as molecules capable of being produced by the host [127]. This predictive approach utilized KEGG pathways present in the host and selected bacteria known to be present in the host GI tract to generate a host-microbiota reaction network model [61]. This *in silico* model was used to predict aromatic amino acid metabolites based on host, microbiota or

cometabolism between host and microbiota. Prediction of compounds was followed with targeted and untargeted LC-MS analysis of intestinal contents from conventional and GF mice to determine the validity of the predictive algorithm and identify metabolites which required a microbial metabolic component for synthesis. This novel approach predicted a set of bioactive amino acid metabolites which required a bacterial component for production and identified two microbiota-generated metabolites (5-hydroxy-L-tryptophan and salicylate) as activators of the AhR [128].

2.2 Obesity

Obesity, defined as a massive expansion of the adipose tissue, is associated with several severe diseases including type 2 diabetes, coronary heart disease and cancer. Diabetes related health cost in 2012 was $245 billion [13], a significant increase from previous annual diabetes related costs in 2007 of $113 billion (2007 dollars) [14]. Obesity related diseases such as type 2 diabetes and coronary heart disease continue to be a serious problem in the modern developed world where the obese population continues to expand.

While dietary, nutritional, environmental, and lifestyle factors are all well established as triggers for the development of obesity [17, 129], the intestinal microbiota has been more recently identified as an important contributor to the onset and progression of obesity as well as obesity-induced diabetes and inflammation [16, 17]. The onset of

obesity results in a pronounced alteration in the microbiota composition and function which has been correlated to alterations in host metabolism, inflammatory state and epithelial barrier function [16, 20, 21]. The microbiota of obese individuals has an increased capacity for energy harvest [17] and reduced bacterial diversity compared to the microbiota of lean individuals [130]. Animals on a diet high in fat had a decreased number of species present in the microbiota due to a large bloom in Mollicutes, a class of Firmicutes [131]. High fat diet fed animals also had a significantly higher relative abundance of the Firmicutes and a significantly lower relative abundance of Bacteroidetes compared to lean fed animals. In fact, several experimental models for investigating insulin resistance utilize a high fat diet to cause the initial weight gain [23]. Gnotobiotic humanized mice shifted from a plant rich polysaccharide diet to a high fat diet display clear shift in the genetic content of the microbiota, including an increase in genes involved in nutrient processing, within a single day [24].

One of the consequences of obesity is an increased permeability of the epithelial layer to bacterial lipopolysaccharide (LPS). The initiation and persistence of inflammation in the liver and adipose tissue has been linked to increased levels of bacterial lipopolysaccharide (LPS) in circulation in obese individuals due to a leaky epithelial barrier in the gut [19].

The microbiota does not contribute toward the progression of insulin resistance in obesity only by altering gut permeability. In an elegant and now classic experiment,

Backhed et al. colonized GF mice with microbiota isolated from the cecum of conventionally raised mice and found that colonization of GF mice by the microbiota increased body mass compared to animals which remained germ free [132]. This increase in body mass was attributed to an increased absorption of monosaccharides from the gut in the presence of the microbiota which led to an increase in lipogenesis in the liver. Analysis of genetically obese mice containing a mutation in the gene encoding leptin (*ob/ob*) identified that *ob/ob* animals had a 50% reduction in the abundance *Bacteroidetes* and an increased population of *Firmicutes* [133]. Ley et al. identified changes in the community structure of obese animals occurred at the division level [133]. More recent studies have probed finer levels of microbial taxonomy. *Bifidobacterium* populations increased in the microbiota of *ob/ob* and high fat diet fed mice from age 7-11 weeks compared to lean controls while *Lactococcus* populations decreased in the microbiota of *ob/ob* mice indicating changes in microbiota populations extend down to the genus level [134]. Turnbaugh et al. conclusively showed that colonization of germ free mice with a human derived microbiome isolated from an obese individual caused a significant increase in fat mass gain over a two week period compared to colonization of GF mice with a lean host derived microbiota [135]. A similar experiment was performed at the strain level by Fei et al. in which GF mice monoassociated with *Enterobacter cloacae B29* isolated from a morbidly obese individual developed obesity on a high fat diet, whereas GF control mice did not develop obesity [136]. Thus, the microbiome is altered in obesity and this altered microbial

community, at the genus and in some instances the strain level, contributes to obesity directly increases fat mass by increasing energy harvest in the host [17, 136].

2.2.1 The adipose tissue

Adipose tissue is composed of adipocytes, immune cells including resident tissue macrophages and T-cells, nerve tissue and stromovascular cells [137]. Adipocytes account for roughly 15% of cells in the adipose tissue with vasculature associated cells accounting for 70% and other cells making up the 15% balance of total cells in adipose tissue, however by sheer size adipocytes account for the 90% of tissue volume in adipose tissue. The primary role of the adipose tissue is regulation of energy storage which is accomplished by adipocytes through storage of energy during lipogenesis and release of energy during lipolysis [138]. During excess food consumption, surplus energy is stored in adipocytes in the form of triglycerides in lipid droplets. It is these lipid droplets give adipocytes their characteristic morphology [139]. During fasting, lipase enzymes convert triglycerides to free fatty acids which are then secreted and serve as fuel for other organs [140].

Once thought of as simply an energy reservoir for the body, adipose tissue is now accepted as a highly active metabolic and endocrine organ [141]. The adipose tissue actively secretes chemokines in response to inflammatory stimuli including interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), monocyte chemoattractant protein 1 (MCP-1),

ACDC and leptin among many other cytokines and adipokines with various paracrine and endocrine functions [141]. The obese state is characterized by chronic, low grade inflammation in the host adipose tissue which has a causative role in the progressive dysregulation of whole-body metabolism. These metabolic alterations include defective insulin signaling[142], increased FFA levels [143] and altered lipid metabolism [144]. The low grade chronically inflamed obese state, driven in part by elevated levels of circulating LPS, eventually causes progressive deterioration of host insulin sensitivity ultimately causing type 2 diabetes [18-20].

One hallmark of obesity is elevated levels of circulating adipose tissue derived cytokines (e.g., TNF-α, IL-6). This increase in circulating cytokines is a response to increased levels of bacterial derived pro-inflammatory LPS [19]. In this model for initiation of inflammation, obesity increases permeability of the epithelial barrier in the gut contributing to a "leaky" gut which allows LPS enters circulation at an increased rate compared to a normal epithelial barrier [19, 20]. In this model, the visceral adipose tissue immediately surrounding the gut is exposed to elevated levels of LPS. The adipocytes in this tissue, as well as the resident tissue macrophages, produce inflammatory cytokines in response to LPS stimulation [145, 146]. These adipocyte and macrophage derived cytokines and chemokines such as TNF-α, IL-6 and ACDC as well as MCP-1 have direct causative roles in the development of persistent chronic inflammation and insulin resistance [18, 29-31]. Due to both chronic LPS exposure and paracrine signaling between adipocytes and macrophages, inflammatory cytokines

persist at elevated levels in adipose tissue in obesity and have been shown to have a causative role in the development of insulin resistance [18, 147].

In-vitro investigations into the interactions between macrophages and adipocytes indicate that co-culture of macrophages with adipocytes increases pro-inflammatory TNF-α gene expression in both cell types and TNF-α, IL-6 and MCP-1 gene expression in adipocytes [148]. Stimulation of adipocytes with TNF-α, which was primarily derived from macrophages in adipocyte-macrophage co-culture experiments, showed a significant increase in MCP-1 cytokine expression. Adipocytes and macrophages co-cultured and stimulated with LPS display increased expression of MCP-1, IL-6 and CXCL-1 in adipocytes compared to cells not stimulated with LPS [149]. The progression of obesity induced inflammation also involves significant accumulation of pro-inflammatory M1-like inflammatory macrophages to the adipose tissue into localized regions called crown like structures [40]. Taken together, both the number and extent of activation of macrophages can affect the inflammatory state of neighboring adipocytes. In the context of obesity, macrophage infiltration and activation state are both classic markers of obesity and insulin resistance [150, 151].

2.2.2 Macrophage polarization

In addition to an increase in cytokine production by adipocytes and resident tissue macrophages, obesity induces a phenotypic switch in adipose tissue macrophages [32].

The macrophage has been traditionally described as a phagocytic cell responsible for performing homeostatic maintenance including the removal apoptotic and senescent cells with other critical roles in activating both the innate and acquired immune response [32, 33]. Due to the differing microenvironments local macrophages experience, resident tissue macrophages have broad roles in homeostatic maintenance of the tissue in which they are present [34]. The phenotypic activation state, or polarization state, of macrophages is a broad area of study describing the roles of macrophage populations in tissue remodeling, inflammation, cancer and obesity [20, 32, 35]. Macrophage polarization is classically defined by three factors which characterize how the macrophage functions in the context of the host tissue; the metabolic macrophage state, the expression of cell surface receptors and the production of functional cytokines [36-39]. Initially the pro-inflammatory M1 activated macrophage state was identified followed later by the M2 or "alternatively" activated macrophage. However, recent evidence has suggested a degree of plasticity between the two extremes initially described as M1and M2 [37, 152]. These definitions of M1 and M2 polarized macrophages were derived from a pre-genomic era of research in which discrete, individual markers were considered sufficient to establish the phenotypic state of the cell. Importantly, while these M1 and M2 states are not rigidly fixed and in fact macrophages *in-vivo* exist in numerous states between M1 and M2 and several confounding mediators can generate intermediate states [152, 153].

Polarization from a naive M0 macrophages *in-vitro* involves stimulation of bone marrow progenitors with cytokine or endotoxin stimulation [37]. The macrophage cytokine production profile in response to LPS stimulation shows a clear bias towards the induction of molecules important in infection response signaling for responding to infection [32]. M1 polarization classically involves either interferon gamma (IFN-γ) or LPS treatment [154]. Production of cytokines such as TNF-α, IL-1β and IL-6 are hallmarks of the classic M1 polarization state of macrophages in response to stimuli such as LPS [155]. LPS stimulation strongly also induces intracellular pathogen defense mechanisms such as the inducible nitric oxide synthase (iNOS) for the production of antimicrobial nitric oxide [156-158].However, extended LPS exposure induces metabolic and phenotypic changes in macrophages which fundamentally alters the macrophage cytokines, cell surface receptor patterns and functional metabolic processes of the cells [37]. Mechanistically, IFN- γ binds to the interferon gamma receptor (IFN-γR) complex which causes dimerization of the IFN- γRα subunit leading to the recruitment of IFN- γRβ [159]. Two janus kinases (JAK), JAK1 and JAK2, are associated with the IFN-γR complex. Upon ligand binding, JAKs phosphorylate IFN-γRα recruiting the signal transducer and activator of transcription 1 (STAT1) and activating the STAT pathway [160]. STAT1phosphorylation induces STAT homo dimerization and nuclear translocation, DNA binding and transcription activation [161].

In addition to IFN-γ, LPS also polarizes macrophages toward a M1 phenotype [162]. LPS binds to LPS binding protein (LBP) which then associates with CD14 in the TLR-4

complex [163]. MyD88 then recruits IL-1 receptor associated kinase 4 (IRAK-4) through interaction with the death domain [164]. TNF receptor-associated factor 6 is then activated (TRAF6), which then complexes with ubiquitin-conjugating enzyme 13 (UBC13) and ubiquitin-conjugating enzyme E2 variant 1 isoform A (UEV1A) to then activate transforming growth factor-β-activated kinase 1 (TAK1). TAK1 then activates IκB kinase (IκK) which then phosphorylates inhibitor of κ light chain gene enhancer in B cells (IκB). IκB then disassociates from nuclear factor-κB (NF-κB) and is ubiquitinated and destroyed. NF-κB then translocates to the nucleus and promotes transcription of genes downstream of the κB binding sites.

The M2, or alternatively activated, anti-inflammatory state is activated by cytokines classically derived from Th-2 cells including interleukin 4 (IL-4), interleukin 10 (IL-10) or interleukin 13 (IL-13) [165, 166]. Upon exposure to IL-4 or IL-10, M0 macrophages develop an altered phenotype which is characterized by changes in cytokine production, metabolism and cell surface receptor patterns and can be identified as M2 polarized macrophages [167]. These M2 polarized macrophages classically have an increased expression of the cytokines found in inflammatory zone 1 (FIZZ-1) and Ym1, cytokines found in tissue remodeling and allergic inflammation, as well as arginase 1 (Arg1), an enzyme involved in tissue remodeling and repair [165, 168, 169].

IL-4 dependent M2 activation, as with M1 activation, is achieved through JAK STAT family of protein pathways [170]. IL-4 binds to the IL-4 receptor (IL-4R) complex,

activating janus kinase 3 (JAK3). The signal transducer and activator of transcription 6 (STAT6) then associates with the IL-4R complex where JAK3 phosphorylates STAT6. STAT6 then homodimerizes and translocates to the nucleus where it binds specific consensus sequences and promotes transcription of downstream genes. IL-10 signaling also utilizes JAK-STAT pathway signaling [171]. IL-10 binds the heterodimeric IL-10 receptor complex containing IL-10 receptors 1 and 2. JAK1 and non-receptor protein-tyrosine kinase 2 (TYK2), which are constitutively associated with the IL-10 receptor complex, then phosphorylate the IL-10 receptor, leading to recruitment and activation of STAT3 [172]. Activated STAT3 then dimerizes and translocates to the nucleus where it binds specific DNA elements and activates transcription [172, 173].

2.2.3 mTOR, the crossroads of obesity and immunity

Obesity is not only a metabolic disorder, but also an immune disorder as the dysregulation of adipose tissue macrophages has a causative role in the progression of insulin resistance and type 2 diabetes [19, 20, 158]. The mTOR pathway is uniquely positioned at the crossroads of host metabolism, amino acid sensing and immune system [43]. The role of mTOR in protein synthesis has been extensively studied. mTORC1 directly phosphorylates the translational regulators eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and S6 kinase 1(S6K1) and thereby activates protein translation [48]. mTOR functions as a key regulator in the induction of autophagy, an essential process in all myeloid and lymphoid cells [174]. *Shigella*

flexneri infection causes cells to undergo amino acid starvation and downregulation of mTOR, triggering autophagy induction which helps clear the infection [175]. Aside from roles in maintaining cellular processes during variable nutrient availability and aiding in defense against pathogens, the mTOR pathway has been implicated in an increasing number of pathological conditions including obesity and type 2 diabetes, cancer and neurodegeneration [52-54].

One crucial, but relatively poorly understood function of mTOR is the regulation of amino acid sensing in the cell. Under amino acid-sufficient conditions, mTORC1 is localized to the lysosome whereas under amino acid starved conditions, mTORC1 localizes throughout the cytoplasm [176].The active RAG GTPase complex RAGA/B·GTP–RAGC/D·GDP has been shown to be required for mTORC1 localization to the lysosome as knockdown of RAG GTPases prevented mTORC1 association with lysosomes [176]. The primary transporter for mTOR dependent amino acid sensing is the SLC7A5/SLC1A5 complex by transport of L-glutamine which is the rate limiting signal in mTORC1 activation [177]. Interestingly, inhibition of mTOR by glutamine starvation upregulates SLC7A5 expression indicating a feedback mechanism within the cell for controlling amino acid uptake once amino acid levels have stabilized [51]. The mTOR signaling pathway in the context of classic amino acid and growth signaling is summarized in **Figure 2**.

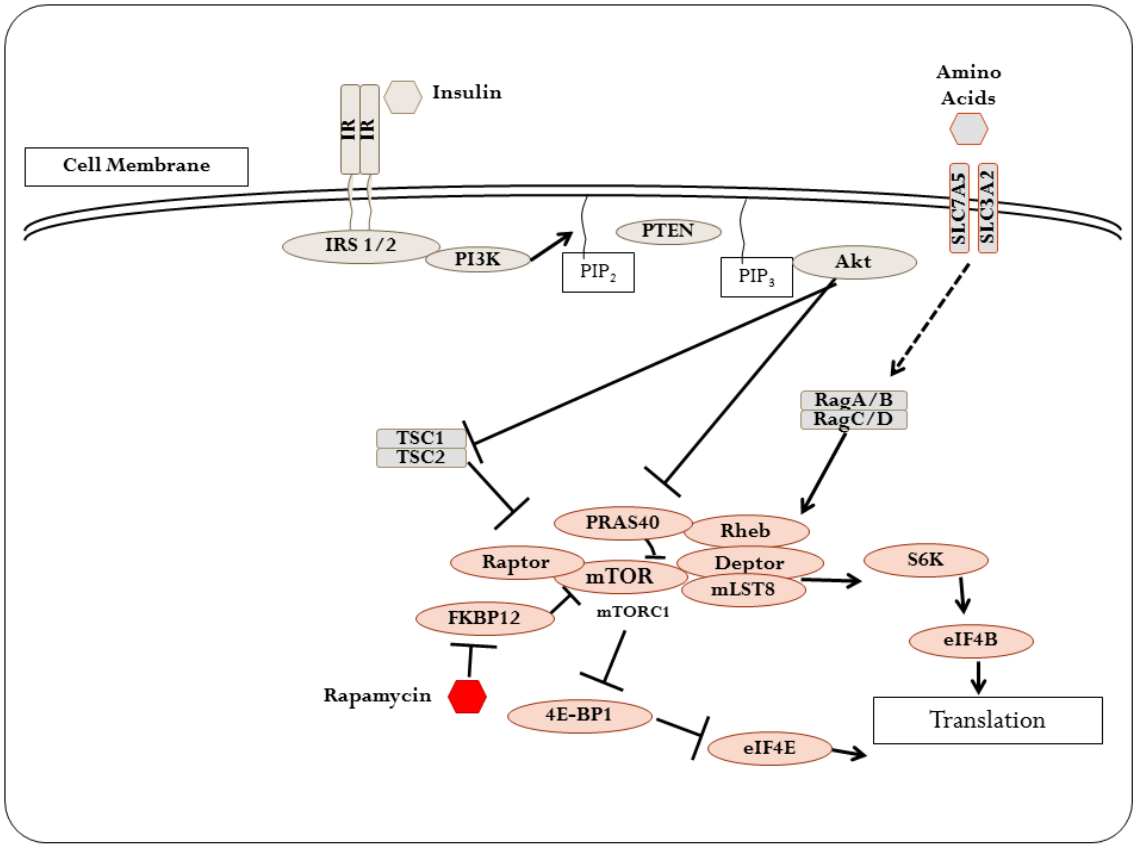


Figure 2. mTOR signaling at a glance. The activated mTOR complex 1 phosphorylates the S6 kinase. S6K then phosphorylates EIF4B which leads to the initiation of translation. Akt phosphorylates and inactivates the TSC1/2 complex relieving the TSC1/2 complex block on mTORC1. Akt also phosphorylates and inactivates PRAS40 causing dissociation of PRAS40 from mTORC1 relieving the PRAS40 block on mTOR activity.

Regulation of mTOR has a central role in macrophage polarization through the tuberous sclerosis proteins (Tsc) 1/2 complex which functions as a negative regulator of mTOR activity [55]. Myeloid specific deletion of Tsc1 led to chronic activation of mTOR in macrophage populations [55]. These Tsc1 deficient macrophage populations were refractory to IL-4 induced M2 polarization signals and displayed increased M1 polarization associated cytokines in response to LPS stimulation. Chronic activation of mTOR also caused a downregulation of Akt signaling which was critical for the altered polarization signaling in Tsc deficient macrophages. Macrophage colony stimulating factor (M-CSF) is the primary cytokine signal responsible for differentiation of monocyte progenitor cells into macrophages [178, 179]. M-CSF binds the cell surface receptor CD115 (M-CSFR, C-FMS) which then transduces a pro-survival signal through the PI3K/Akt/mTOR pathway [178]. This intersection between pro-survival signals through one arm of the mTOR pathway and nutrient sensing in the form of amino acid availability through the other arm of the mTOR pathway elegantly illustrates the diverse range of processes under the control of mTOR signaling.

Several classes of molecules have been used for modulation of mTOR signaling. Rapamycin is commonly used in the clinic to modulate mTOR signaling to promote immunosuppression and prevent rejection of organs after transplantation [180]. Several other FDA approved mTOR inhibitors (also called rapalogs) such as temsirolimus, everolimus and deforolimus are also used in the clinic for treatment of renal cell carcinoma (tersirolimus) and hematologic and solid malignancies (everolimus) [181]. In

addition to these exogenous or synthetic compounds, several naturally derived molecules containing the indole moiety have also been shown to alter mTOR signaling. For example, the plant-derived indole-3-carbinol (I3C) and diindolylmethane (DIM), a dimer of I3C produced that is spontaneously formed from acid degradation products of I3C, inhibit the phosphorylation and activation of Akt to inactivate mTOR [182, 183], and have been proposed as potential therapeutic molecules against prostate cancer [184-186].

2.3 The aryl hydrocarbon receptor

The AhR is an intracellular ligand-activated nuclear receptor [187]. The AhR is a cytosolic receptor which exists in its latent state as part of a complex including the known complex-associated proteins heat shock protein 90 (hsp90), the tyrosine kinase c-src and hepatitis B virus X-associated protein 2 (XAP2) [188]. This small molecule activated receptor was initially identified as the receptor for TCDD [189]. Ligand binding to the AhR causes conformational changes to the AhR protein and cofactor disassociation [190]. This release of AhR from its cofactors exposes the nuclear localization signal and causes translocation of the ligand-AhR complex to the nucleus [188]. Upon entering the nucleus, HSP90 and XAP2 dissociate from the ligand-bound AhR which activates binding of aryl hydrocarbon receptor nuclear translocator (ARNT) to the ligand-bound AhR. The AhR-ARNT complex then binds to dioxin response elements (DREs) and activates transcription of AhR responsive genes such as the

prototypical AhR target gene cytochrome P4501A1 (CYP1A1). AhR ligands include several compounds from the halogenated aromatic hydrocarbon category of contaminants including 3,3',4,4',5-pentachlorobiphenyl, indole containing molecules including 6-Formylindolo[3,2,-b]carbazole and diindolylmethane and polyphenols including curcumin and quercetin [190]. AhR is primarily expressed in the lung with an approximate expression level of 30 molecules / 100ng RNA, with the heart, liver, thymus and brain having 5-10 fold lower expression levels than the lung [191]. The AhR is expressed in pre-adipocyte fibroblasts but expression of AhR decreases during adipogenesis and in pre-adipocytes the AhR negatively regulates adipogenesis [192, 193]. Classical AhR signaling is summarized in **Figure 3**.

The importance of the AhR in host immunology is rapidly expanding. The AhR regulates the differentiation of IL-17 producing T(T_h17) and T_{reg} cells [128],and subsequently has been shown to have roles in autoimmune diseases including AhR dependent development of Th17 cells promoting experimental autoimmune encephalomyelitis (EAE) [194]. The AhR negatively regulates LPS activation of TLR-4 dependent inflammatory responses in macrophages [195]. AhR deficiency in macrophages leads to increased production of IL-6 and TNF-α in response to LPS treatment and AhR-deficient mice are more susceptible to LPS-induced lethal shock than WT mice [195]. This suggests AhR acts as an essential negative regulator of the LPS signaling pathway and promotes survival in LPS-induced shock models of infection and points to a role for the AhR in host fitness [196]. The However, the AhR also influences

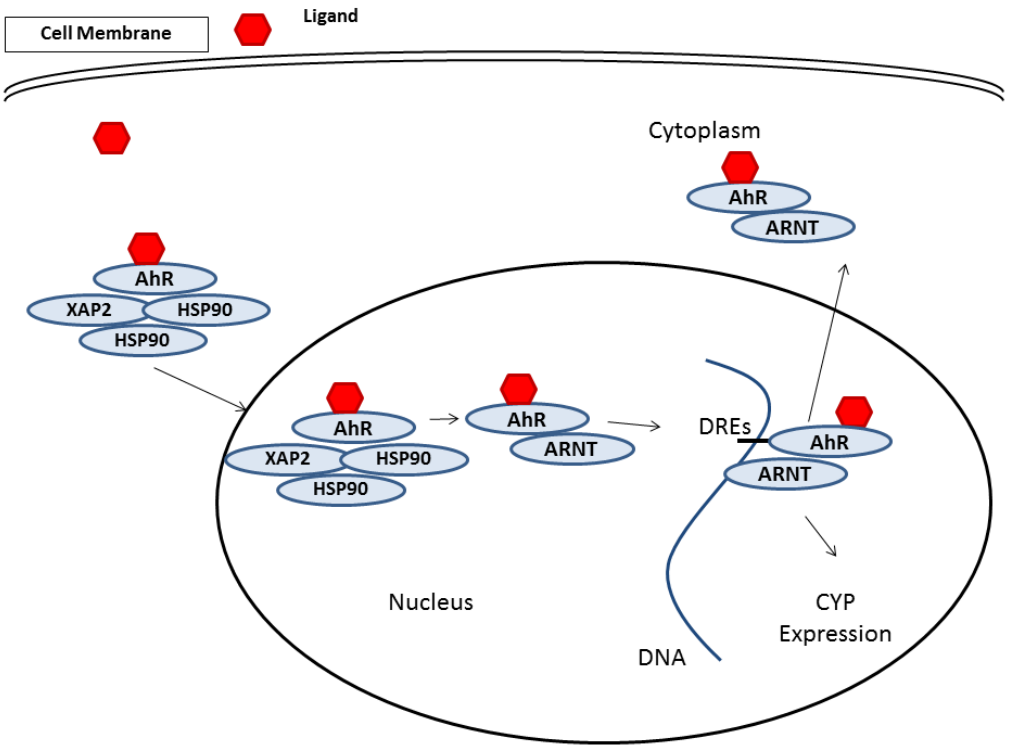


Figure 3. Schematic of AhR signaling. AhR ligands cross the cell membrane and bind to cytosolic AhR in the AhR complex comprised of AhR, XAP2 and HSP90. The ligand bound complex then translocates to the nucleus where the AhR-ligand complex dissociates from XAP2 and HSP90 and binds ARNT. The AhR-ligand-ARNT complex then binds to DREs and activates transcription of AhR response genes such as CYP1A1.

activation of dendritic cells as TCDD treatment induces AhR dependent activation of dendritic cells indicating exposure to the environmental contaminant TCDD alters activation of the immune system [197]. Additionally, LPS and CpG induce AhR expression in bone marrow derived dendritic cells (BMDCs). The AhR therefore has an immense and diverse role in activation and development of immune cells where it plays key roles as an anti-inflammatory factor in macrophages and as a pro-inflammatory factor in T cells.

Recent work has identified several endogenous ligands of the AhR including kynurenine, 7-ketocholesterol, bilirubin, indole and other indole-like molecules [61, 71, 72, 198]. The tryptophan microbial derived metabolites indole functions a partial agonist/antagonist AhR of the AhR in CaCo-2 and MDA-MB-468 cells [72]. Indole-3-acetate and tryptamine, both indole containing molecules derived from tryptophan, act as AhR agonists and activate Ah-responsive genes including CYP1A1. Indole itself has been shown to bind the human AhR in both *in vitro* binding experiments as well as *in silico* ligand docking simulations [71]. The enzyme indoleamine 2,3-dioxygenase (IDO) is the rate limiting enzyme in the conversion of tryptophan to kynurenine [199]. Endogenous tryptophan catabolism through IDO and its product kynurenine have been established as important for the development of the immune system via activation of the AhR [67]. In a disease tolerance model in mice, initial LPS challenge activated AhR and IDO, the enzyme responsible for production of the AhR ligand kynurenine, and activated AhR was able to downregulate early gene expression [196]. Upon a second LPS

challenge, AhR negatively downregulated inflammatory gene expression only in the presence of IDO [67]. Interestingly, AhR positively regulates IDO expression and subsequent kynurenine production specifically in in dendritic cells, a key cell type involved in cytokine production in LPS induced shock models [200].

2.4 Surface receptors

The cell surface is covered in receptor proteins capable of binding [201], transporting [177], and transducing signals [202], from a diverse array of ligands including small molecules including lipopolysaccharides [203], other proteins [201], other cells [204], viruses [205] and a myriad of ligands too diverse to list. Microbial derived metabolites activate a variety of cell surface and intracellular host receptors. The FFA receptors including g-protein coupled receptors GPR40, GPR41, GPR43, GPR84 and GPR120 recognize nonesterified fatty acids of varying sizes [69]. SCFA's such as butyrate have an essential role in activating regulatory colonic T-cell populations and colonic Tregs are activated through the G coupled protein receptor (GPR) 43 [70, 206]. This work conclusively showed that the products of the microbiota were not only sensed by host immune cells, but that SCFA's regulate colonic Treg homeostasis through GPR43.

Identifying surface receptor-ligand binding interactions, as are involved in protein-protein binding, often involves rigorous and laborious experiments utilizing combinations of classical techniques including immunoprecipitation [207] or yeast two

hybrid screens [208]. Pappas et. al identified a membrane localized estrogen receptors (ER) for the molecule estradiol by a combination of techniques including covalently conjugating estrogen to bovine serum albumin (BSA) to prevent estrogen from crossing the cell membrane [209]. This work was later confirmed in a separate report utilizing competition binding studies between estradiol and highly purified estradiol-BSA to the cells expressing a membrane estrogen receptor but not a nuclear estrogen receptor, conclusively showing estradiol binds to both a membrane ER and nuclear estrogen receptors ERα and ERβ [201, 210]. Similar approaches have been adapted to other small molecules to prove the ligand activates a cell surface receptor without crossing the cell membrane [211, 212].

3. MATERIALS AND METHODS

3.1 Reagents

Murine 3T3-L1 fibroblasts were kindly provided by Prof. Barbara Corkey (Boston University School of Medicine, Boston, MA). RAW264.7 cells were obtained from ATCC (Manassas, VA). Dulbecco's Modified Eagle Medium (DMEM), RPMI-1640, insulin and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). Bovine serum and fetal bovine serum (FBS) were obtained from Atlanta Biologicals (Flowery Branch, GA). Ultrapure LPS derived from *S. minnesota* was obtained from Invivogen (San Diego, California). Indole was obtained from Sigma Aldrich (St. Louis, MO). Optima grade solvents were obtained from Fisher Scientific (Pittsburg, PA). Optima grade solvents were obtained from Fisher Scientific (Pittsburg, PA). Simply Blue Safe Stain was obtained from Invitrogen.

3.2 Animals

Male 20 week old C57BL/6J mice fed 60% fat diet for 12 weeks and control mice fed 10% fat diet for 12 weeks were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free animal facility at Massachusetts General Hospital. Female 6-8 week old C57BL/6J and C57BL/6-Ahr$^{tm1.2Arte}$ (AhR$^{-/-}$) mice were purchased from Taconic Biosciences (Hudson, NY) and maintained in a specific

pathogen-free animal facility located at Texas A&M University Health Science Center. All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines under an approved animal use protocol.

3.3 Extraction and quantitation of indole from serum

The concentration of indole in serum from low-fat and high-fat diet mice was determined by mass spectrometry (MS). Metabolites were extracted from serum using a methanol extraction method with minor modifications [213]. Briefly, 200 µL of ice-cold methanol was added to 50 µl serum. Samples were vortexed and incubated at -20 ^{o}C for 1 h. The sample was centrifuged at 15,000 × *g* for 10 min at 4 ^{o}C. The supernatant was then transferred to a clean tube and centrifuged at 15,000 × *g* for 10 min at 4 ^{o}C. The supernatant was collected, dried by solvent evaporation in an Eppendorf speedvac concentrator (Eppendorf, Hauppauge, NY), and reconstituted in 50 µL of methanol/water (1:1, v/v). Extracted metabolites were stored at -80 ^{o}C until analysis.

Indole was detected and quantified on a triple quadrupole linear ion trap mass spectrometer (3200 QTRAP, AB SCIEX, Foster City, CA) coupled to a binary pump HPLC (Prominence LC-20, Shimazu, Concord, Ontario, Canada) as previously described [72, 214]. Chromatographic separation was achieved on a hydrophilic interaction column (Luna 5 µm NH_2 100 Å 250 mm × 2 mm, Phenomenex, Torrance,

CA) using a solvent gradient method [215]. Peak identification and integration were performed using Analyst software (version 5, Agilent, Foster City, CA).

3.4 Adipocyte culture and differentiation

Standard differentiation of adipocytes was performed as previously described [216]. Briefly 3T3-L1 adipocytes were grown in pre-adipocyte growth medium consisting of DMEM (high-glucose; 4.5 g/l) supplemented with calf serum (10%, v/v), penicillin (200 U/ml), and streptomycin (200 μg/ml). During this period, medium was replenished every other day. On day 2 after confluence, the cells were induced to differentiate using an adipogenic cocktail consisting of 1 μg/ml insulin, 2 nM 3,3′,5-triiodo-L-thyronine (T3), 0.5 mM isobutylmethylxanthine (IBMX), and 1 μM dexamethasone added to a basal medium (DMEM with 10% FBS and penicillin/streptomycin). After 48 h, the medium was replaced with a second induction medium consisting of the basal adipocyte medium supplemented with only insulin and T3. After another 48 h, the medium was replaced with the basal adipocyte medium. Medium was replenished every other day through day 10 after induction.

3.5 Macrophage culture

RAW264.7 macrophages were cultured in DMEM supplemented with 10% FBS, penicillin (200 U/ml), and streptomycin (200 μg/ml) at 37 °C/5% CO_2. Bone marrow

derived macrophages (BMDM) were isolated from the femur of mice as previously described [217]. Briefly, femurs were isolated and cut open, and a 23-gauge needle was used to flush out the marrow with ice-cold media. The marrow was centrifuged and the cell pellet resuspended in ACK lysis buffer for 5 min to lyse red blood cells [218]. An equivalent amount of RPMI supplemented with 10% FBS, penicillin (200 U/ml), and streptomycin (200 μg/ml) (RPMI-C) was added to halt lysis. Cells were centrifuged, resuspended in complete RPMI supplemented with 10 ng/mL M-CSF (Invivogen, San Diego, California, USA) and passed through a 70 μm cell strainer. Cells were seeded in polystyrene dishes in RPMI + M-CSF and incubated for 3 days at 37 °C/5% CO_2. On day 3 the RPMI + M-CSF medium was replenished and cells were incubated for an additional 4 days. At the end of the 7-day period, cells were 95% positive for macrophage markers F4/80 and CD11b.

3.6 Flow cytometry analysis

The expression of TNF-α under different experimental conditions was determined using flow cytometry as previously described [65]. Briefly, RAW264.7 cells were cultured as indicated in the presence of GolgiPlug protein transport inhibitor (BD Biosciences, San Jose, CA), fixed, then stained with a fluorescence-tagged TNF-α antibody (BD Biosciences) diluted 1:200. In the case of BMDMs, macrophages were first co-stained with a fluorescence-tagged CD11b antibody (Abcam, Cambrige, MA) diluted 1:200, then fixed and stained as above.

The expression of phosphorylated proteins under different experimental conditions was determined using flow cytometry. BMDM's were cultured and surface stained with a fluorescence-tagged CD11b antibody (Abcam), then fixed as above. Cells were permeabilized with methanol on ice for 20 minutes prior to phosphoprotein staining. Cells were then co-stained with a p-mTOR (ABCam) antibody diluted 1:500 and then stained with a fluorescence-tagged rabbit IgG (ABCam) antibody diluted 1:200.

The expression of macrophage cell surface proteins under different experimental conditions was measured using flow cytometry. BMDM's were cultured as above and surface stained with a F4/80 (eBiosciences, San Diego, California) antibody diluted 1:200 followed by a fluorescence-tagged-streptavadin conjugate diluted 1:5000, CD11b (eBiosciences), a fluorescence-tagged CD11c(BioLegend, San Diego, California) antibody diluted 1:200 and a fluorescence-tagged CD206 (BioLegend) antibodies diluted 1:200.

Stained cells were subjected to FACS analyses using a BD FACSAria II or BD LSRFortessa flow cytometer (BD Biosciences) that is operated by Texas A&M Health Science Center College of Medicine Cell Analysis Facility. Data (10,000 events per sample) were analyzed by using FlowJo software (Tree Star, OR).

3.7 RNA extraction and qRT-PCR analysis

Gene expression in adipocytes or macrophages was carried out using quantitative reverse transcription-PCR (qRT-PCR). After cells were treated as described in each experimental condition, cell supernatants were gently removed and cells were washed with phosphate-buffered saline (PBS). PBS was then remove and cell pellets were stored at −80 °C before RNA extraction and analysis. RNA extraction and purification was performed using the EZNA Total RNA kit (Omega Bio-Tek, Norcross, GA). RNA was quantified using SYBR green based qRT-PCR (qScript One-Step PCR kit; Quanta Biosciences, Gaithersburg, MD) on a Light Cycler 96 (Roche, Indianapolis, IN). Primers are listed in Table 1.

3.8 Generation of conditioned media

Macrophage stimulated media (MSM) was generated using RAW 264.7 macrophages. Cells were seeded in 10 cm dishes, and stimulated with 10 μg/mL LPS for 4 h. Cells were then washed with PBS and incubated with DMEM for an additional 12 h. MSM was harvested and sterile filtered using a 0.22 μm filter and stored at -80 °C until use. Adipocytes were stimulated on day 10 post differentiation with MSM diluted 1:2 with adipocyte maintenance media.

Table 1. RT-PCR primers used in the study

Gene	Primer Sequence
18S_F	GAGGCACTCCCCCAAAAGAT
18S_R	TCTAAGGGCATCACAGACCT
IL-6_F	TCCAGTTGCCTTCTTGGGACTGAT
IL-6_R	AGCCTCCGACTTGTGAAGTGGTAT
MCP-1_F	CTCTCTTCCTCCACCACCAT
MCP-1_R	ACTGCATCTGGCTGAGCCA
TNFα_F	TCTCATGCACCACCATCAAGGACT
TNFα_R	TGACCACTCTCCCTTTGCAGAACT
IL1β_F	TCCAGGATGAGGACATGAGCAC
IL1β_R	GAACGTCACACACCAGCAGGTTA
CXCL1_F	ACCCAAACCGAAGTCATAGCC
CXCL1_R	TTGTCAGAAGCCAGCGTTCA
CD206_F	GATATGAAGCCATGTACTCCTTACTGG
CD206_R	GGCAGAGGTGCAGTCTGCAT
Arg1_F	CAGAAGAATGGAAGAGTCAG
Arg1_R	CAGATATGCAGGCAGGGAGTCACC
FIZZ1_F	CCTGCTGGGATGACTGCTAC
FIZZ1_R	CAGTGGTCCAGTCAACGAGT
iNOS_F	GGAGCCTTTAGACCTCAACAGA
iNOS_R	AAGGTGAGCTGAACGAGGAG
YM1_F	CATTAAATGGTCTGAAAGACAGGA
YM1_R	TCTGAGGAGTAGAGACCATGGAA

Adipocyte stimulated media (ADSM) was generated using 3T3-L1 adipocytes at day 10 post-differentiation as described above. Cells were stimulated with 10 µg/mL LPS for 12 hours. Cells were then washed with PBS and incubated with RMPI-C for an additional 12 hours. ADSM was harvested and sterile filtered using a 0.22 um filter and stored at -80 °C until use.

3.9 Macrophage migration

Migration of BMDM cells were carried out in transwell cell culture chambers with polycarbonate membranes (24 well, 8 µm pore, Corning Costar). BMDM cell suspensions were added to the upper chamber with or without medium containing indole for 4 h. After pre-incubation, medium in both chambers was removed and media with or without indole was replaced in the top chamber. Media with or without 20 ng/mL MCP-1 (Invitrogen, Carlsbad, CA, USA) and indole was then added to the bottom chamber. After incubation for 24 h, cells remaining on the upper side of the membrane were scraped off using a cotton swab. The remaining migrated cells were fixed with methanol for 15 min. Fixed cells were stained with 0.1% crystal violet for 30 min. Stained cells were counted under Axiovert 200M invert microscope (Zeiss) using a 10x objective. Ten fields were chosen randomly per well for counting and three replicate wells were analyzed in each experiment. BMDM migration in response to ADSM was performed as indicated above with ADSM diluted 1:10 with RPMI-C.

3.10 Glucose uptake assay

BMDM's were pre-treated with indole for 4 hours. After pre-treatment, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) was added at to final concentration 10 uM for 2 hours while also maintaining the indicated concentrations of the indole derivatives. Cells were then fixed and subjected to FACS analysis as described above.

3.11 Nitric oxide measurement

Nitric oxide was measured from cell supernatants using a commercially available Greiss Reagent Kit (Promega, Madison, WI) according to the manufacturer's instructions.

3.12 Conjugation of indole-3-carboxaldehyde with bovine serum albumin

Indole-3-carboxaldehyde (I3CA) was used instead of indole to conjugate to BSA because indole does not have a reactive group to which BSA can be conjugated to for the selected conjugation chemistry. Conjugation of I3CA to BSA to generate indole-3-BSA (I3-BSA) was carried out using a 3 step reaction scheme (**Figure 4**). BSA was dissolved in phosphate buffered saline (PBS) with 2 mM ethylenediaminetetraacetic acid (EDTA). Traut's reagent was added at a molar excess and incubated with mixing for 1 h at room temperature to convert free amines on BSA to sulfhydryl groups. Excess Traut's reagent

was removed using a Zeba 7K MWCO spin column (ThermoFisher, Waltham, MA) at 1000 x g for 2 minutes at room temperature. The sulfhydryl containing BSA was then reacted with a 10-fold molar excess of N-κ-maleimidoundecanoic acid hydrazide (KMUH) with mixing for 2 h at room temperature to react the sulfhydryl group with the maleimide forming a thioether bond. Unreacted KMUH reagent was removed using a Zeba 7K MWCO spin at1000 x g for 2 minutes at room temperature. I3CA was added at a 10-fold molar excess with mixing for 2 h to react the hydrazine containing BSA with the carboxaldehyde to form a hydrazone bond between indole and conjugate BSA. Unreacted I3CA was removed using a Zeba 7K MWCO spin 1000 x g for 2 minutes at room temperature. The final protein concentration was measured using the BCA Assay and used to estimate the concentration of the I3-BSA conjugate. The I3-BSA conjugate was desalted and concentrated using an Amicon Ultra-15 3 kDa centrifugal filter (EMD Milipore, Billerica, MA).

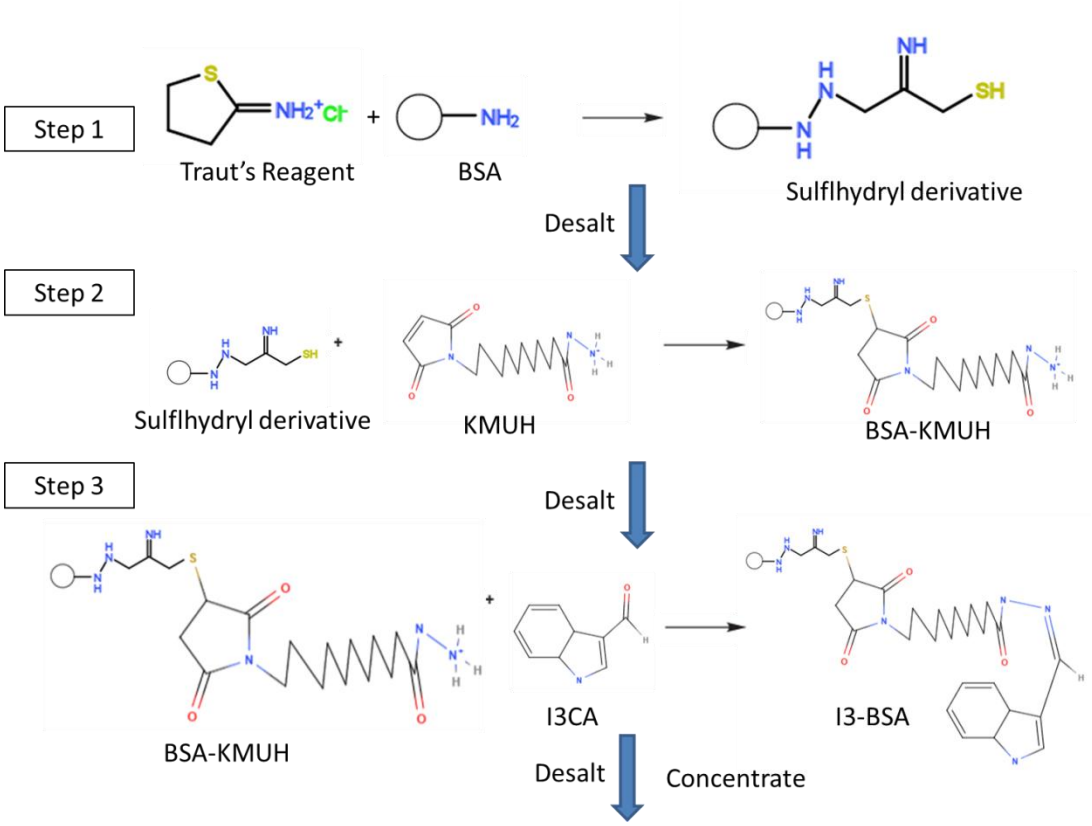


Figure 4. I3-BSA 3 step reaction schematic.

4. DETERMINE THE EFFECT OF INDOLE ON THE ADIPOCYE AND MACROPHAGE INFLAMMATORY RESPONSE

4.1 Introduction

Studies have shown that physical interactions between GI tract bacteria and host cells are important for maintaining the host epithelium [56, 57]. In this work, we investigated whether indole's anti-inflammatory effects extend beyond the intestinal epithelial cells to adipocytes and macrophages. Specifically, we investigated if indole attenuates cytokine and chemokine production and macrophage chemotaxis as indicators of inflammation in adipocytes and macrophages. The increased level of bacterial LPS in circulation due to an increase in host gut permeability in the obese model is a trigger for adipose tissue inflammation in the surrounding adipose tissue [19]. Both macrophages and adipocytes produce inflammatory cytokines in response to LPS stimulation and these LPS induced cytokines have been shown to contribute to the progression of insulin resistance. Therefore, we hypothesized that indole in circulation could attenuate the LPS induced production of inflammatory cytokines in both macrophages and adipocytes. We further investigated if indole's effect in these cell types is mediated through the AhR. Our results suggest indole and other tryptophan-derived microbiota metabolites could be potent modulators of inflammation in sites distal from the GI tract.

4.2 Detection of indole in murine serum

Our prior work revealed the presence of indole in murine gut cecal contents which was dependent on the microbiota [72, 214]. Therefore, we first determined if indole is also present in serum and if its levels are altered in an experimental model of obesity. The serum indole concentration from mice reared on a low-fat (10%) diet (LDF) and a high-fat (60%) diet (HFD) model of obesity was quantified using mass spectrometry. **Figure 5** shows that serum from mice on the low-fat diet contained ~ 1 μM indole whereas mice on a high-fat diet had ~ 50% less indole ($p < 0.1$). The presence of indole in serum indicates that indole does reach organs in the host distal to the gut and the concentration of indole is lower in animals fed a diet high in fat.

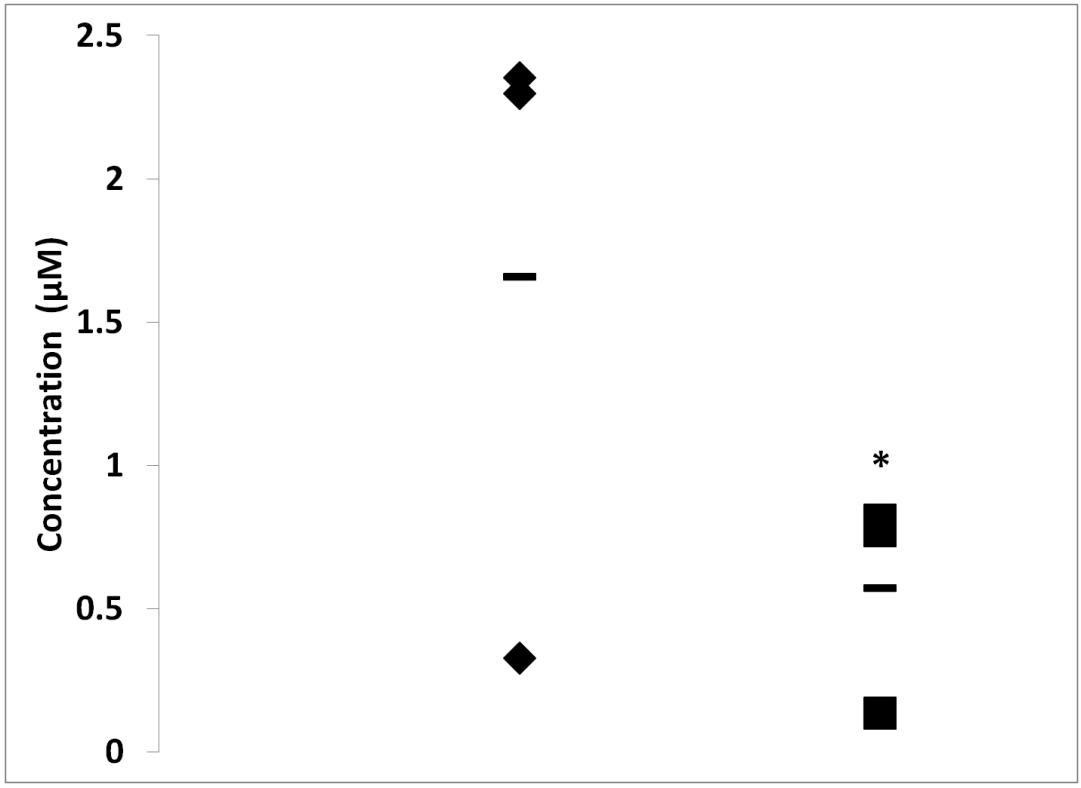
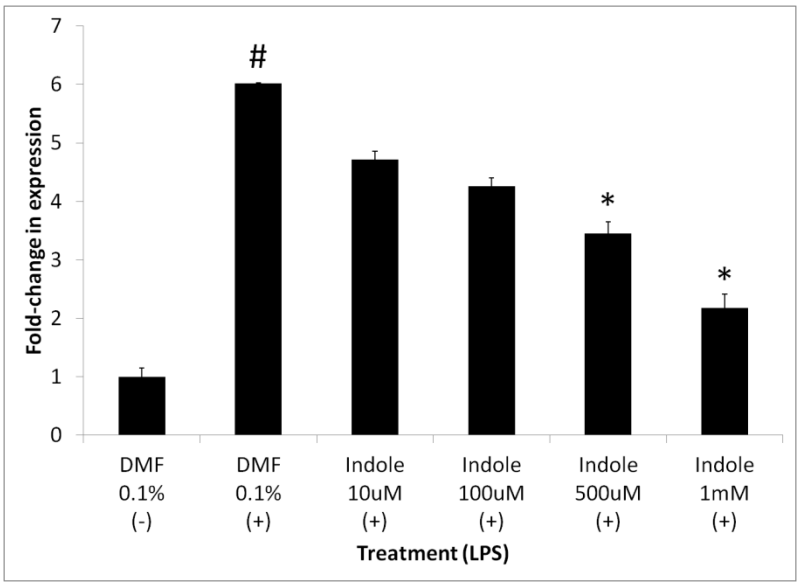


Figure 5. Quantitation of indole in serum from low-fat diet control and high-fat diet mice using LC-MS. Serum was collected from the two experimental groups (n = 3 each) and metabolites extracted using methanol extraction. Indole was detected using MRM-mass spectrometry. Pure indole was used to generate a standard curve (0 – 50 µM) and used to quantify indole concentration in the different samples. * indicates statistical significance at using the Student's t-test at $p < 0.1$.

4.3 Indole attenuates macrophage TNF-α production upon LPS stimulation

The increased level of bacterial LPS in circulation due to an increase in host gut permeability in the obese model is a trigger for adipose tissue inflammation leading to the production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β by the adipose tissue macrophages [19]. Prolonged exposure to gut bacterial derived LPS has been shown to have a causative role the development of insulin resistance [18, 147]. Therefore, we measured the effect of indole in attenuated the LPS-mediated production of inflammatory cytokines in macrophages. RAW264.7 cells were exposed to different concentrations of indole ranging from 10 μM to 1 mM for 4 hours, and then stimulated with LPS in the continued presence of indole. **Figure 6A** shows that macrophages exposed to indole (500 μM and 1000 μM) had a 3-fold decrease in TNF-α gene expression compared to the solvent treated stimulated control and this effect was evident in a dose-dependent manner. However, indole did not attenuate the expression of IL-6 or IL-1β at any concentration tested. The attenuation of LPS-induced alterations in TNF-α by indole was also investigated at the protein level. RAW264.7 cells pre-treated with indole prior to LPS stimulation resulted in dose-dependent reduction in the population of cells positive for TNF-α production (**Figure 6C**). Indole's inhibitory effect was significant at all concentrations tested with the lowest effective concentration at 10 μM and a maximum reduction of 50% at an indole concentration of 1 mM.

A



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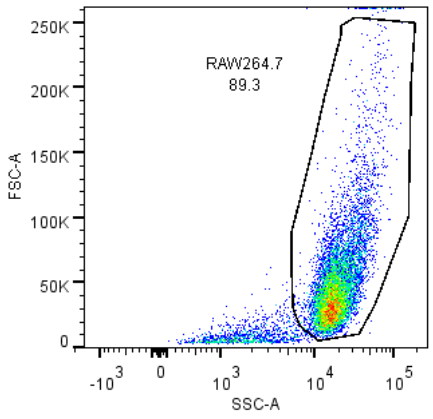

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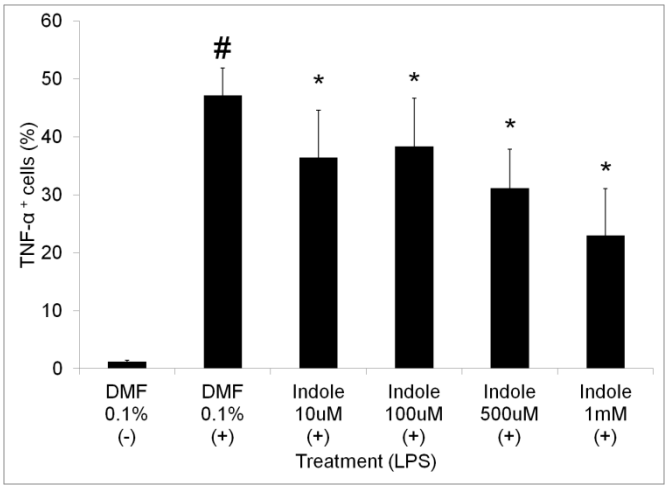


Figure 6. (A) Effect of indole on macrophage TNF-α gene expression in response to 250 ng/mL LPS as measured by qRT-PCR. RAW264.7 cells were treated with indole for 4 h followed by exposure to LPS for 6 h. (B) RAW264.7 gating strategy for FACS analysis showing forward scatter by side scatter. (C) Effect of indole on macrophage TNF-α accumulation in response to LPS stimulation as measured by ICS-FACS. Data shown are from three biological replicates per experiment and two independent experiments. "-/+" indicates absence or presence of LPS. "#": statistical significance between LPS-stimulated and unstimulated control or "*" between indole-treated and solvent stimulated using the Student's t-test at $p < 0.05$.

4.4 Indole inhibits macrophage migration to MCP-1

Since one of the hallmarks of obesity is increased infiltration of macrophages into adipose tissue [219], we examined whether macrophages exposed to indole demonstrated altered migration to chemoattractants *in vitro*. BMDM's were utilized in an unpolarized state rather than the RAW264.7 cell line as previous reports have indicated that RAW264.7 cells are present in an activated state and do not respond to chemoattractants [220]. BMDM's treated with medium containing the monocyte chemoattractant protein-1 (MCP-1) demonstrated approximately 2-fold increase in migration compared to controls **(Figure 7**). Exposure to 100 μM and 500 μM indole prior to MCP-1 stimulation significantly decreased macrophage migration down to the level of unstimulated (i.e. no MCP-1) BMDM's. Furthermore, exposure to 1 mM indole completely abolished migration to MCP-1 **(Figure 7**).

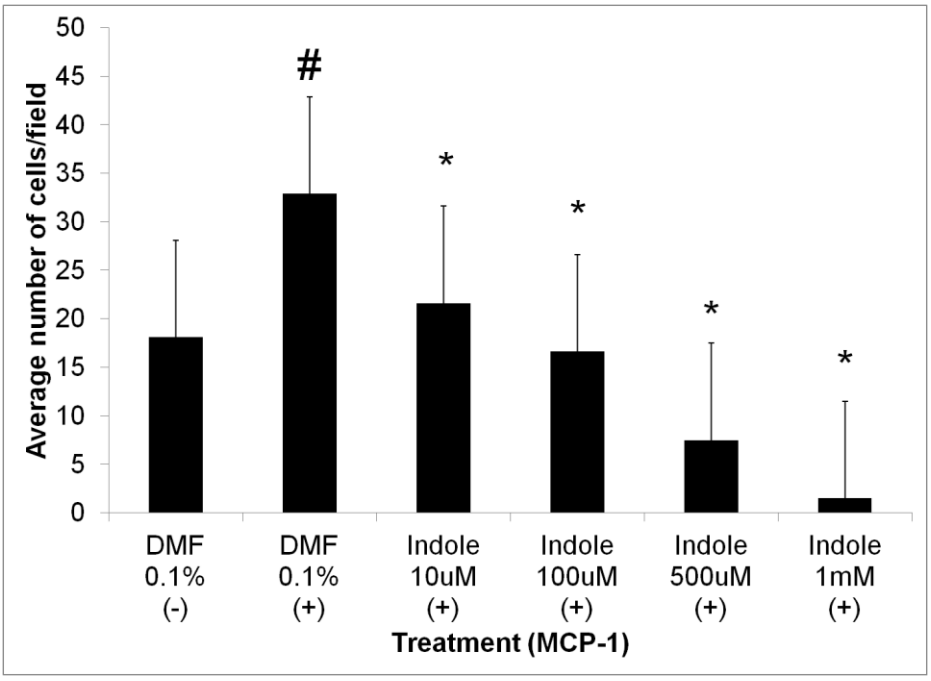
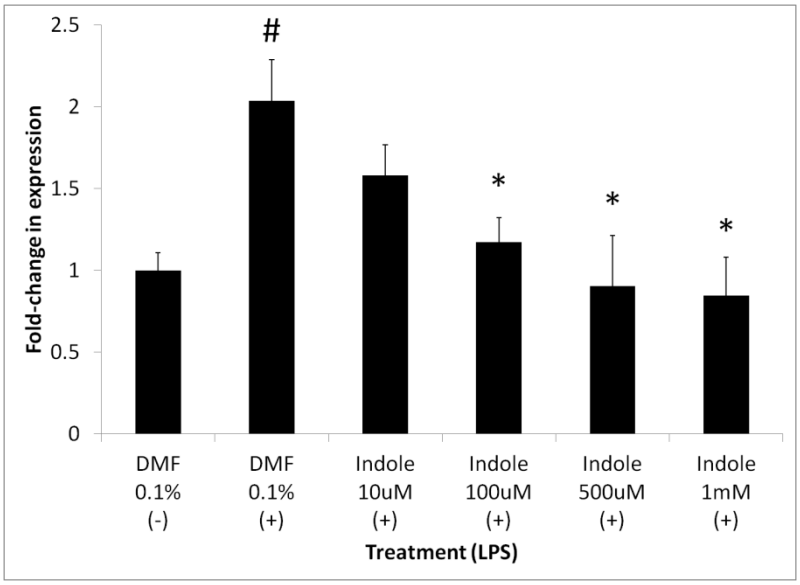


Figure 7. Effect of indole on macrophage migration in response to 20 ng/mL MCP-1. BMDM's seeded in transwell chambers were treated with indole for 4 h followed by exposure to MCP-1 for 24 h. Cells which migrated to the bottom of the transwell membrane were counted in in 10 individual fields per transwell. Data shown are from three biological replicates of BMDMs per experiment obtained from two independent isolations. "-/+" indicates absence or presence of MCP-1. "#": statistical significance between stimulated and unstimulated control or "*" between indole-treated and solvent stimulated using the Student's t-test at $p < 0.05$.

4.5 Indole attenuates adipocyte MCP-1 and IL-6 expression

Adipocytes are the primary source of MCP-1 in adipose tissue in response to circulating LPS [221]; therefore, we investigated if indole also inhibited LPS-mediated induction of MCP-1 gene expression in 3T3-L1 adipocytes. Adipocytes pre-treated with indole for 4 h and then stimulated with LPS for an additional 6 h showed approximately 10-fold decrease in MCP-1 gene expression (**Figure 8**). We also quantified the effect of indole exposure on LPS induction of IL-6 gene expression in adipocytes as IL-6 is a major adipocyte-produced cytokine involved in obesity related adipose tissue inflammation [222]. **Figure 8** shows that indole attenuated IL-6 gene expression in a dose-dependent manner, indicating indole attenuates both expression of cytokines and chemokines involved in obesity related adipose tissue inflammation.

A



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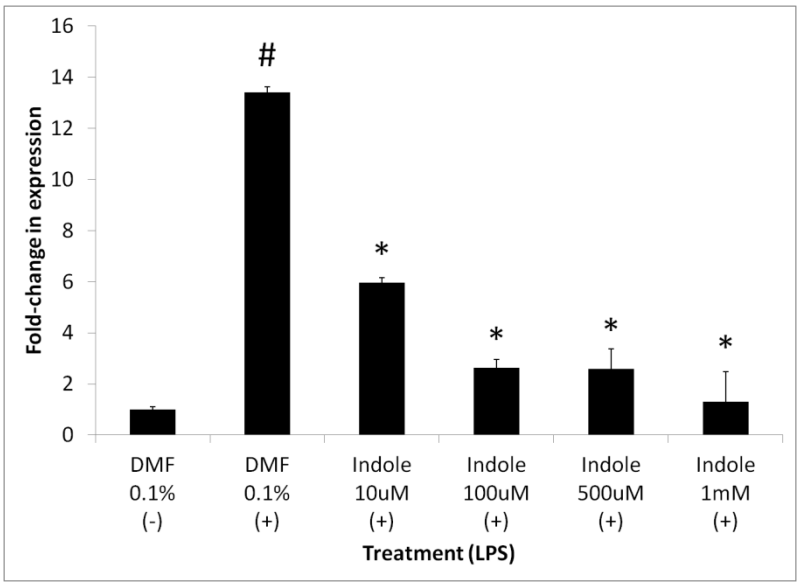

Figure 8. Effect of indole on adipocyte expression of IL-6 (A) and MCP-1 (B) in response to 250 ng/mL LPS as measured by qRT-PCR. 3T3-L1 derived mature adipocytes were treated with indole for 4 h followed by exposure to LPS for 6 h. Data shown are from three biological replicates per experiment and two independent experiments. "-/+"indicates absence or presence of LPS. "#": statistical significance between stimulated and unstimulated control or "*" between indole-treated and solvent stimulated using the Student's t-test at $p < 0.05$.

In order to further understand the effect of indole on adipocyte activation, we determined whether indole could inhibit a single macrophage derived cytokine stimulus in adipocytes. Since indole attenuated TNF-α production in macrophages, we also investigated if indole also inhibited TNF-α mediated induction of gene expression in adipocytes. 3T3-L1 adipocytes were pre-treated for 4 h with indole then stimulated with 20 ng/mL TNF-α for 6 h. **Figure 9** shows that indole-treated adipocyte MCP-1 and IL-6 gene expression is similar to indole-untreated after stimulation with TNF-α. This result underscores the fact that MSM is a complex mixture of cytokines and suggests that indole may attenuate the inflammatory effect another cytokine present in MSM.

4.6 Indole attenuates macrophage spent medium activation of adipocytes

Macrophages and adipocytes have a well-established paracrine signaling loop in inflamed obese adipose tissue [148]; therefore we investigated if indole attenuated macrophage-dependent cytokine expression in adipocytes. Mature 3T3-L1 adipocytes were pre-treated with indole for 4 h and then exposed to MSM for 6 h in the continued presence of indole. Indole attenuated the MSM induced expression of the chemokines CXCL-1 (**Figure 10A**) and MCP-1 (**Figure 10B**). However, indole did not alter the MSM induction of IL-6 gene expression in adipocytes (**Figure 10C**). Together with the attenuation of BMDM migration towards MCP-1 by indole, these results strongly suggest a role for indole in reducing the migration of macrophages into adipose tissue.

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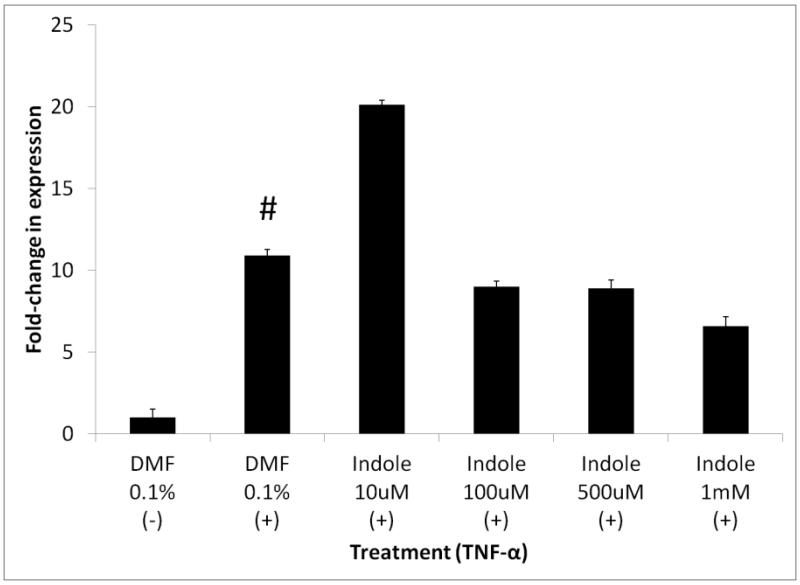

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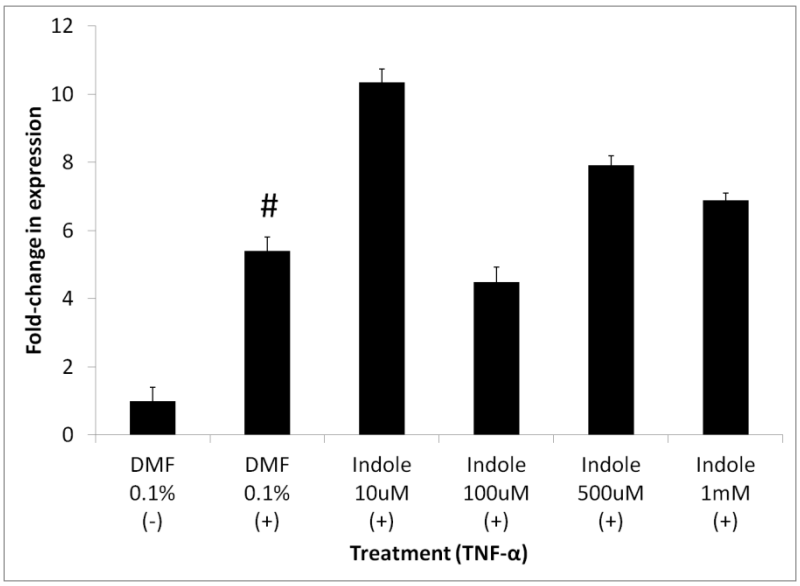

Figure 9. Effect of indole on adipocyte expression of CXCL1 (A) and IL-6 (B) in response to 20 ng/mL TNF-α as measured by qRT-PCR. 3T3-L1 derived mature adipocytes were treated with indole for 4 h followed by exposure to TNF-α for 6 h. Data shown are from three biological replicates per experiment and two independent experiments. "-/+" indicates absence or presence of TNF-α. "#": statistical significance between LPS-stimulated and unstimulated control $p < 0.05$.

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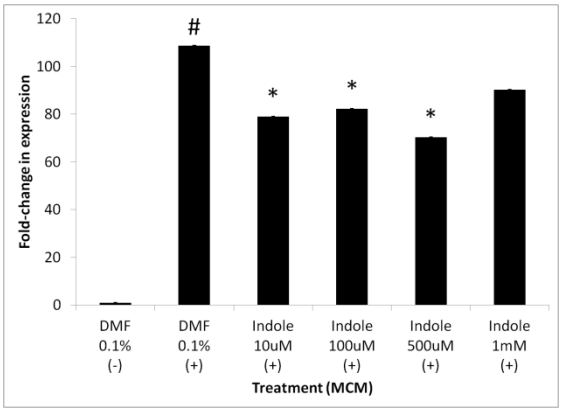

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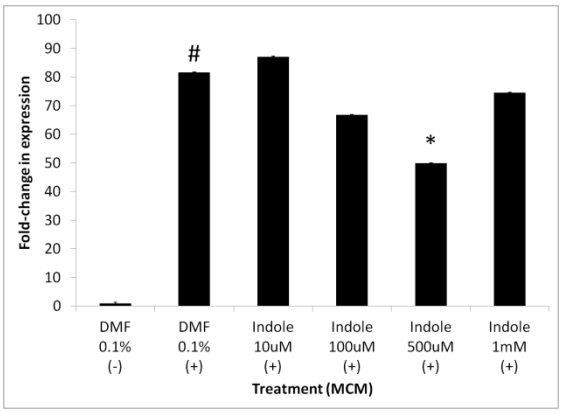

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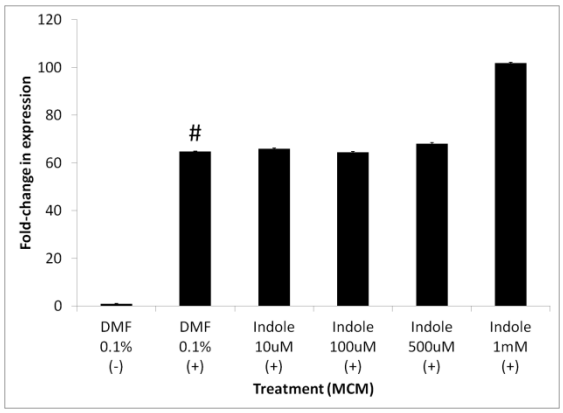

Figure 10. Effect of indole on adipocyte expression of CXCL-1 (A), MCP-1 (B) and IL-6 (C) in response to MSM as measured by qRT-PCR. 3T3-L1 derived mature adipocytes were treated with indole for 4 h followed by exposure to MSM for 6 h. Data shown are from three biological replicates per experiment and two independent experiments. "-/+" indicates absence or presence of MSM. "#": statistical significance between stimulated and unstimulated control or "*" between indole-treated and solvent stimulated using the Student's t-test at $p < 0.05$.

4.7 Indole attenuates macrophage migration to adipocyte spent medium

To further elucidate the role of indole in adipocyte-macrophage paracrine communication [148], we investigated the effect of indole treatment on BMDM migration towards cell-free adipocyte spent medium (ADSM). BMDM's were pre-treated with indole for 4 h, then exposed to ADSM in the continued presence of indole. BMDMs treated with ADSM demonstrated approximately 2-fold increase in migration compared to controls an indole concentration of 500 μM (**Figure 11**). At a concentration of 1 mM, indole completely abolished migration in response to ADCM (**Figure 11**). These results are similar to those obtained using MCP-1 to induce migration (**Figure 7**), and suggest indole modulates inflammatory macrophage-adipocyte paracrine signaling by inhibiting macrophage migration.

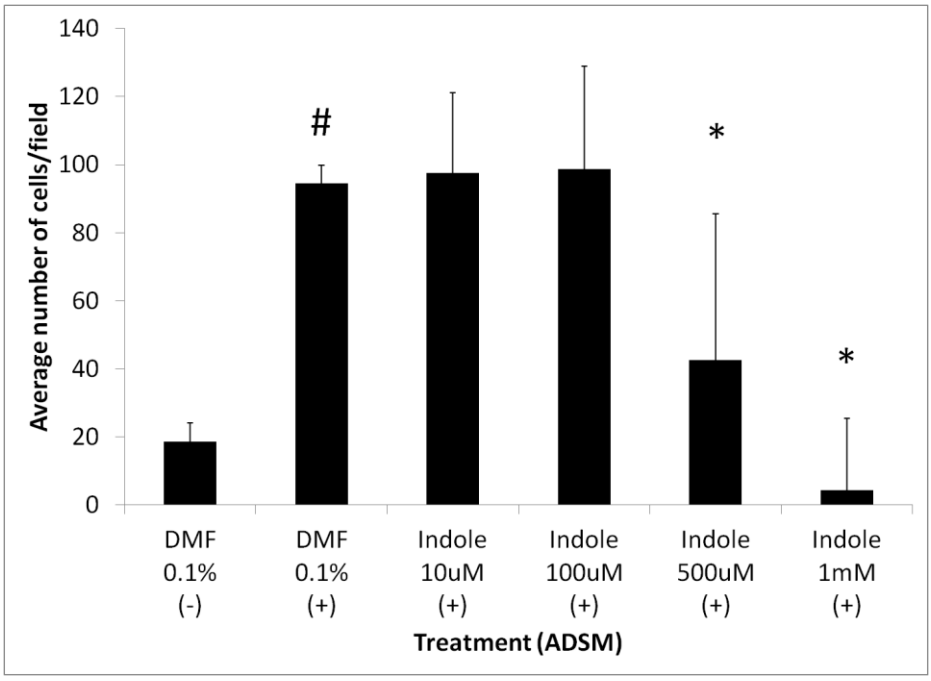

Figure 11. Effect of indole on macrophage chemotaxis in response to ADSM as measured by migration across permeable membranes. BMDM's seeded in transwell chambers were treated with indole for 4 h followed by exposure to ADSM for 24 h. Cells which migrated to the bottom of the transwell membrane were counted in in 10 individual fields per transwell. Data shown are from three biological replicates of BMDMs per experiment obtained from two independent isolations. "-/+" indicates absence or presence of ADSM. "#": statistical significance between stimulated and unstimulated control or "*" between indole-treated and solvent stimulated using the Student's t-test at $p < 0.05$.

4.8 Role for the AhR in indole-mediated attenuation of inflammation

Recent work from our lab demonstrated that indole is a partial agonist/antagonist of the AhR in CaCo-2 and MDA-MB-468 cells [72]. Therefore, we investigated if the attenuation of inflammation in macrophages by indole was mediated through the AhR. BMDM's from $AhR^{-/-}$ mice were pre-treated with indole and the migration in response to MCP-1 treatment was monitored. (**Figure 12**) shows that no significant differences in migration to MCP-1 were observed between the WT and $AhR^{-/-}$ macrophages, suggesting that indole's effects on macrophage migration did not require AhR signaling.

To further understand the role of the AhR in mediating the effects of indole, BMDMs from WT and $AhR^{-/-}$ mice were exposed to different concentrations of indole for 4 h and then stimulated with LPS in the continued presence of indole for 24 h. **Figure 13** shows that exposure to 500 μM and 1 mM indole reduced TNF-α expression in WT BMDMs by approximately 25%. A similar reduction was observed in $AhR^{-/-}$ macrophages as well. These results extend the previous result identifying indole attenuates LPS induction of the inflammatory response in the transformed RAW264.7 cell line (**Figure 6**) to a primary cell model for macrophages which more closely replicates classic macrophage functions including phagocytic activity, cytokine production and oxidative burst compared to cell lines [223]. Together with the macrophage migration studies, these results strongly suggest that the anti-inflammatory effect of indole in macrophages is independent of the AhR.

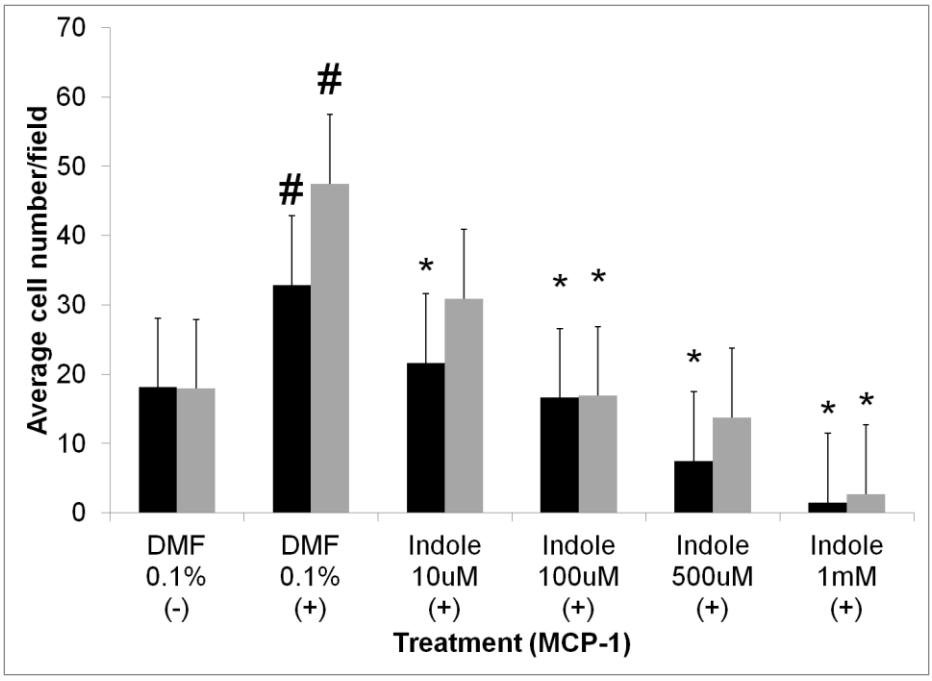

Figure 12. Effect of indole on BMDM chemotaxis in response to 20 ng/mL MCP-1 as measured by migration across permeable membranes. WT (black bars) or $AhR^{-/-}$ (gray bars) BMDM's were treated with indole for 4 h followed by exposure to MCP-1 for 24 h. Cells which migrated to the bottom of the transwell membrane were counted in in 10 individual fields per transwell. Data shown are from three biological replicates of BMDMs per experiment obtained from two independent isolations. "-/+" indicates absence or presence of MCP-1. "#": statistical significance between stimulated and unstimulated control or "*" between indole-treated and solvent stimulated using the Student's t-test at $p < 0.05$.

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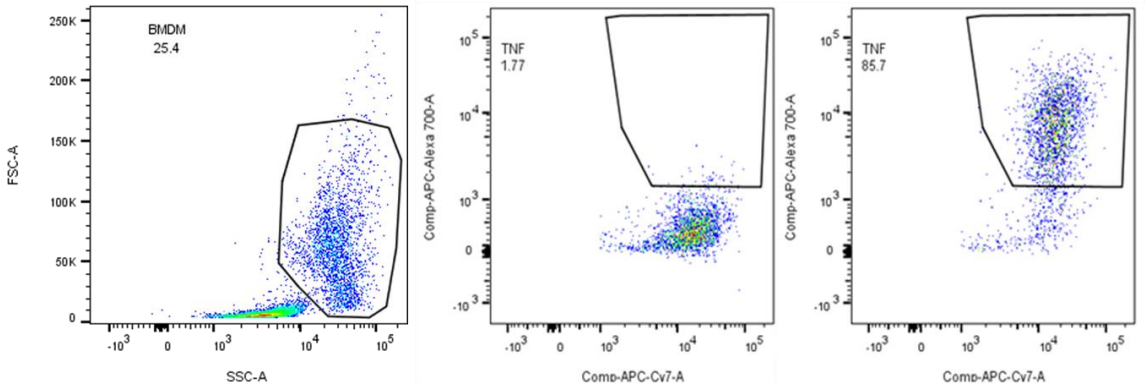

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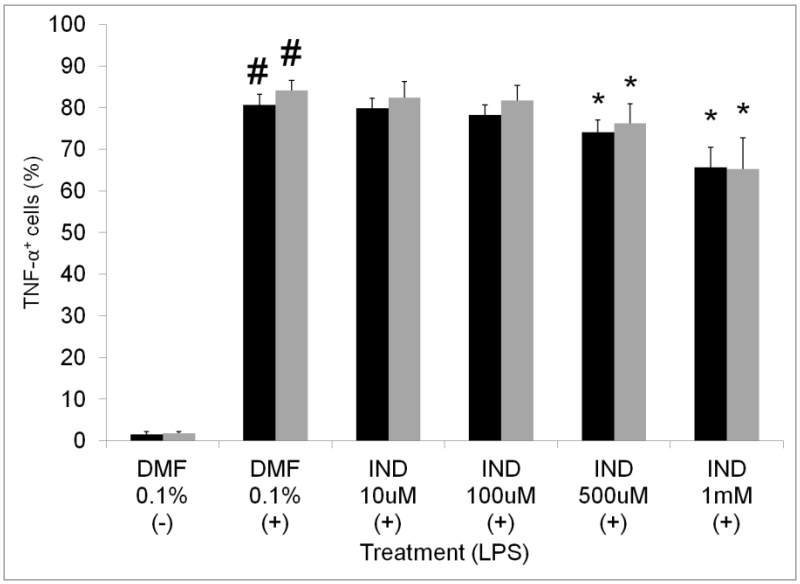


Figure 13. A) BMDM gating strategy for FACS analysis showing forward scatter by side scatter. Also shown are CD11b$^{(+)}$ by TNF-α$^{(+)}$ for both LPS (-) and LPS (+) stimulated conditions. B) Effect of indole on macrophage TNF-α production in response to LPS stimulation as measured by ICS-FACS. WT (black bars) or AhR$^{-/-}$ (gray bars) BMDM's were treated with indole for 22 h followed by exposure to 50 ng/mL LPS for 4 h. Data shown are from three biological replicates of BMDMs per experiment obtained from two independent isolations. "-/+" indicates absence or presence of LPS. "#": statistical significance between stimulated and unstimulated control or "*" between indole-treated and solvent stimulated using the Student's t-test at $p < 0.05$.

4.9 Summary

In this chapter, we investigated whether indole's anti-inflammatory effects extended beyond the GI tract. We specifically focused on adipocytes and macrophages as low-grade chronic inflammation in the adipose tissue is a characteristic feature of obesity and leads to insulin resistance [26, 224]. Since indole has been shown to be present in colonic contents at high concentrations [64, 225] and indole-derived metabolites have been identified in circulation [226], we investigated if indole is also present in murine serum. Our data show that indole is present in murine serum at a concentration of approximately 10 μM, which indicates that indole can reach organs distant from the GI tract.

In addition to modulating LPS-induced inflammation in adipocytes and macrophages, indole also attenuated MSM induced inflammation in adipocytes and ASM induced migration in macrophages. While indole was effective in reducing the response to MSM, it did not attenuate the adipocyte response to TNF-α. This observation could be due to the fact that MSM contains both pro and anti-inflammatory cytokines, and while indole does not attenuate TNF-α signaling, it may modulate other cytokine signaling pathways (e.g., anti-inflammatory cytokines) in adipocytes, which results in the overall reduced inflammatory response observed with MSM. Our data clearly demonstrates that indole inhibits several facets of adipose tissue inflammation, all of which have previously been shown to be involved in the development of insulin resistance [227].

5. ELUCIDATING THE MECHANISM UNDERLYING INDOLE'S ANTI-INFLAMMATORY EFFECT IN MACROPHAGES

5.1 Introduction

Our results in Chapter 4 suggest that the modulation of LPS-mediated inflammation by indole in macrophages is not mediated through the AhR. Therefore, it was of interest to identify the receptor(s) and signaling pathways that are required for indole signaling. Since the AhR is a nuclear (i.e., intracellular) receptor which translocates to the nucleus upon activation by ligand binding [99], we first investigated if indole requires to be internalized (either through simple diffusion or through active transport) for it to be functional in macrophages or whether it can bind to a cell surface receptor and initiate signaling. A search of the literature for known cell surface proteins with small molecule interactions identified the cluster of differentiation 98 (CD98) cell surface complex, composed of SLC7A5 and SLC3A2 [100], could potentially bind to and be involved in transporting indole into cells due to its known function in the uptake of the structurally similar aromatic amino acid tryptophan [101].

Moreover, the CD98 receptor complex has a well-established feedback mechanism with the TSC-mTOR pathway, where the latter has been extensively shown to control the outcome of macrophage polarization [42, 46, 102]. Upon sensing low amino acid levels, mTOR phosphorylation is reduced leading to the transcription of SLC7A5 [42]. This leads to an increase in cell surface expression of SLC7A5 which increases the rate of

amino acid uptake in the cell and thereby phosphorylating mTOR and closing the feedback loop [101]. Therefore, we hypothesized that indole's activity required transport through SLC7A5 at the cell surface to activate a downstream pathway.

5.2 Characterization of I3-BSA conjugate

Since there could be several mechanisms through which indole can be internalized (e.g., involvement of a transporter other than SLC7A5, phagocytosis, diffusion) in macrophages, we first determined whether indole's observed anti-inflammatory effects were required it to be internalized. A classic experimental approach to prevent a molecule from being internalized by a transporter or through the cell membrane by diffusion is to conjugate the small molecule to a larger molecule which cannot diffuse or otherwise pass across the cell membrane [102]. We chose BSA as the conjugate molecule as it is significantly larger than indole (~67 kDa vs 138 Da). Moreover, a similar approach has been used to identify the cell surface receptor for estrogen as described earlier.

The mass of the I3-BSA conjugate was compared to BSA using SDS-PAGE. **Figure 14** shows that the conjugate had a slightly higher molecular weight than the BSA precursor (~67 kDa) which suggests that multiple indole moieties are likely conjugated to a single BSA molecule. The reaction stoichiometry used favors the attachment of multiple indole moieties to BSA. Due to the presence of 121 reactive amine groups based on the sequence of BSA it is likely that individual BSA proteins contain more than one indole moiety with the associated flexible linker.

5.3 I3-BSA conjugate attenuates LPS induction of TNF-α

RAW264.7 macrophages were pretreated with I3-BSA for 4 h prior to stimulation. Cells were then stimulated with 250 ng/mL LPS for 6 h in the continued presence of I3-BSA then fixed, stained for TNF-α and subjected to FACS analysis. Interestingly, the I3-BSA conjugate attenuated TNF-α production (as determined from the % of TNF-α positive cells) upon LPS stimulation by 80% compared to LPS stimulation without I3-BSA treatment (**Figure 15**). The effect of I3-BSA was superior to that observed with indole or I3CA alone (**Figure 15**). This result suggested that the modulation of LPS-mediated inflammation by indole did not require internalization.

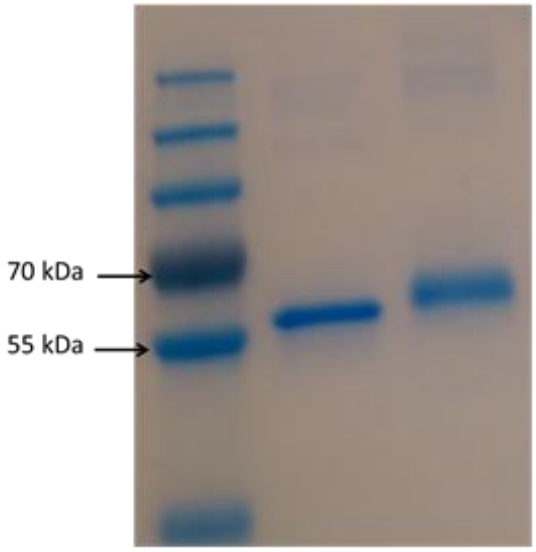


Figure 14. SDS-PAGE analysis of 100 ng of BSA and I3-BSA conjugate loaded per lane onto a 8-16% acrylamide gel stained with Coomassie G-250. Lane 1 (Ladder), lane 2 (BSA), lane 3 (I3-BSA).

5.4 I3-BSA attenuation of TNF-α is independent of phagocytosis

While the above result suggested that I3-BSA was likely not internalized, it is possible that the conjugate could still be internalized through phagocytosis in macrophages. Therefore, we used chemical inhibitors of phagocytosis and endosomal acidification to determine if the attenuation of TNF-α upon LPS stimulation was still observed. The effect of I3-BSA was independent of macrophage phagocytosis, as neither prevention of endosomal acidification with chloroquine nor inhibition of phagocytosis with cytochalasin D altered the effect of I3-BSA (**Figure 15**). These results show the I3-BSA conjugate functions in a manner consistent with indole and I3CA (**Figure 15**). These results further suggest that I3-BSA need not be internalized to exert the observed anti-inflammatory effect, although degradation of the conjugate and direct uptake of unconjugated indole cannot be exhaustively excluded as a possible alternative interpretation of these data. However, it is clear the I3-BSA conjugate functions more effectively than either indole or I3CA.

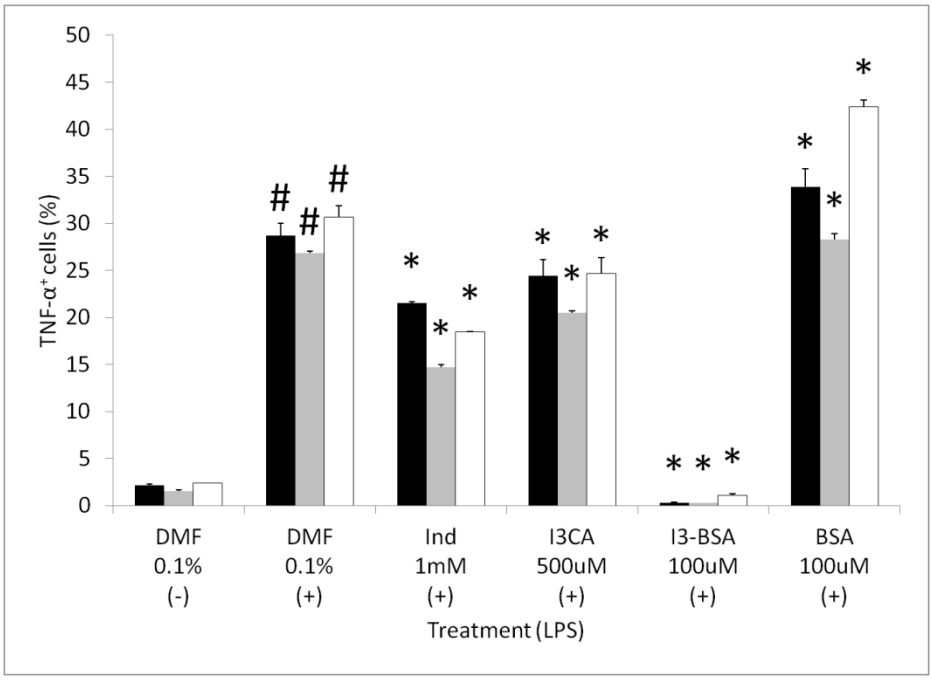


Figure 15. Effect of I3-BSA conjugate on macrophage TNF-α accumulation in response to 250 ng/mL LPS stimulation as measured by ICS-FACS. Cells were co-treated with either 20 µM Chloroquine (gray bars) or 5 µM Cytochalasin D (white bars) or media alone (black bars) in addition to indole derivatives where indicated. Data shown are from three biological replicates per experiment and two independent experiments. "-/+" indicates absence or presence of LPS. "#": statistical significance between stimulated and unstimulated control or "*" between treated and solvent stimulated using the Student's t-test at $p < 0.05$.

5.5 Localization studies with indole-protein conjugate

Our data suggest that I3-BSA does not enter the cell, yet the I3-BSA conjugate leads to a more pronounced inhibition of TNF-α production upon LPS stimulation, as compared to indole or I3CA. These results suggest that the indole conjugate, and by extension indole, may interact with a cell surface receptor and initiate intracellular signaling to modulate inflammation. Therefore, we utilized a fluorescent indole conjugate to determine if the conjugate could be visualized as localized on the cell membrane. We synthesized an indole green fluorescent protein (GFP) conjugate (I3-GFP) using purified recombinant GFP and a reaction scheme identical to that used for synthesizing the I3-BSA conjugate. I3-GFP incubated with RAW264.7 cells for up to 16 h to determine if the fluorescent conjugate could be detected bound to a cell surface receptor. Confocal microscopy visualization shows that despite extended incubation with the I3-GFP conjugate, we could not detect I3-GFP localized to the cell surface (**Figure 16 panel B**). This lack of I3-GFP cell surface interaction could be due to low affinity interaction between the indole moiety and the interacting cell surface molecule, which is consistent with the relatively high concentrations of indole required for attenuating effects in cells. However, it was interesting to note that no fluorescence could be detected inside the cells as well (**Figure 16 panel C**), which further supports the hypothesis that the indole conjugate is not internalized.

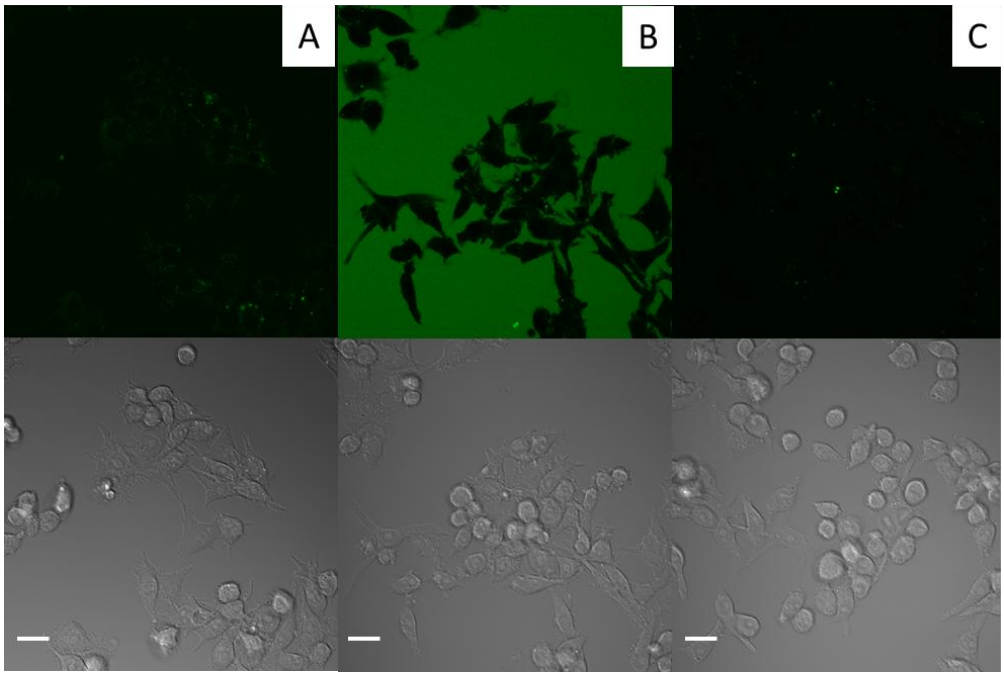


Figure 16. RAW264.7 live cell images. A) Background fluorescence of untreated cells, B) cells in the presence of I3-GFP containing media or C) fluorescence of cells after removing and washing I3-GFP containing media. Scale bar, 50 µm.

5.6 Role of SLC7A5/CD98 complex

We further investigated the role of the SLC7A5 amino acid transporter in the observed modulation of TNF-α signaling upon LPS stimulation. The amino acid transport functions of SLC7A5 have been previously described to be inhibited by the compound 2-aminobicyclo-(2,2,1) heptanecarboxylic acid (BCH) [101, 103]. To test whether SLC7A5 inhibition would alter indole's effect, RAW264.7 cells were treated with BCH at a concentration of 1mM, exposed to different concentrations of indole ranging from 10 μM to 1 mM for 4 h, and then stimulated with LPS in the continued presence of indole. **Figure 17** shows that BCH treatment did not attenuate LPS induction of TNF-α alone and when combined with indole, BCH treatment augmented the effect of indole alone from 22% cells stained positive for TNF-α to 12% cells stained positive with combined treatment. This result was consistent with our results using the I3-BSA conjugate, and suggested that preventing the uptake of indole into the cell increased the attenuation of TNF-α upon LPS stimulation. BCH's augmentation of indole's effect suggested a link between the effect of indole and CD98, possibly that indole is being transported across the cell membrane by the CD98 complex and once inside the cell, is either degraded by the cell or inactive due to no longer being able to interact with an external cell surface receptor. While it is possible that indole uptake can be carried out using other transporters, it is likely that indole modulates macrophage inflammation without entering the cell similar to the I3-BSA conjugate.

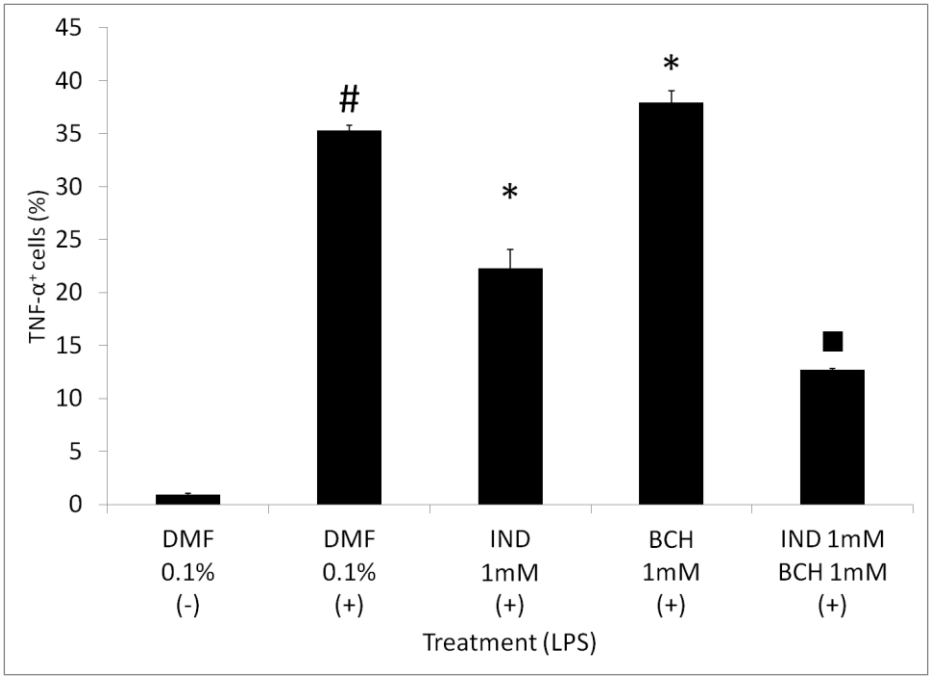


Figure 17. Effect of CD98 blockage on LPS induction of TNF-α. TNF-α accumulation in response to 250 ng/mL LPS stimulation as measured by ICS-FACS. Data shown are from three biological replicates per experiment and two independent experiments. "-/+" indicates absence or presence of LPS. "#": statistical significance between stimulated and unstimulated control, "*" between treated and solvent stimulated or "■" between indole-treated alone and indole co-treated with BCH using the Student's t-test at $p < 0.05$.

5.7 Indole alters mTOR signaling

Since the CD98 complex has been shown to be involved in natural killer (NK) cell metabolism [228] and mTOR-dependent development of NK cells, we investigated the effect of indole on the mTOR signaling pathway by directly measuring the phosphorylation of mTOR (S2448) in BMDMs. BMDMs were used rather than RAW264.7 cells due to the fact that transformed cell lines often have aberrant mTOR signaling and highly polarized M1 macrophages, such as the RAW264.7 cell line, also have highly altered mTOR activities [229-232]. BMDMs were exposed to indole, rapamycin (a known mTOR inhibitor; [181]), luteolin (a PI3K/Akt inhibitor; [233]), or LY294002 (a mTOR and PI3K inhibitor; [234]), for 22 hours then fixed and stained for phosphorylated mTOR and subjected to FACS analysis.

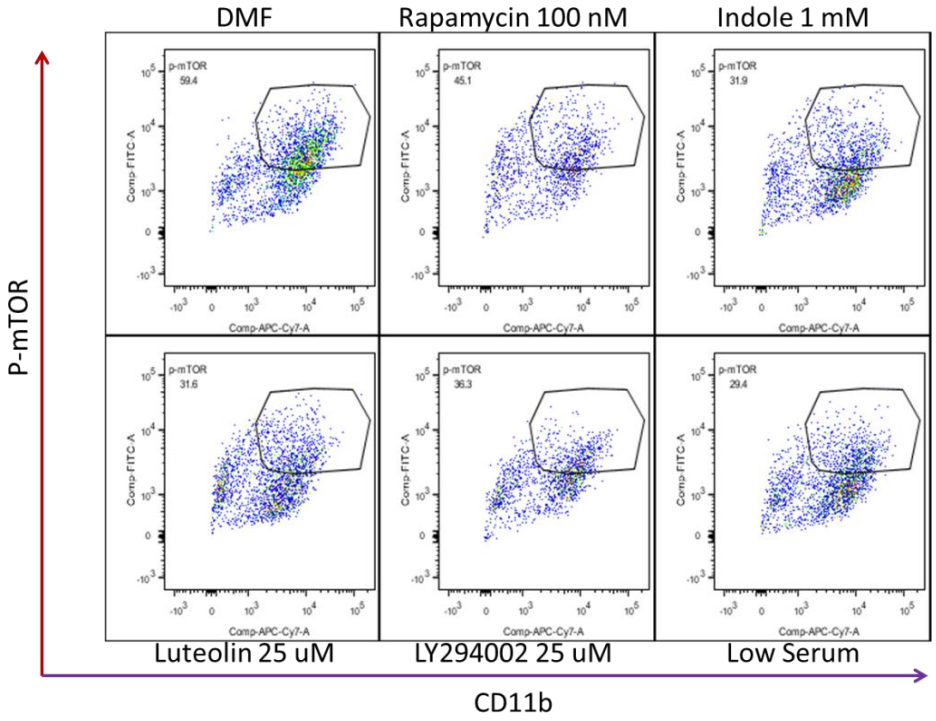
Figure 18A clearly shows indole reduced the percentage of cells positive for phosphorylation of mTOR by 45% at a concentration of 1 mM. Importantly, the mean fluorescent intensity (MFI) of cells stained for p-mTOR was also decreased in cells treated with indole indicating that the amount of phosphorylated mTOR within individual cells was reduced in indole treated cells. As expected, the positive controls Luteolin (LUT) and LY294002 (LY) also significantly reduced mTOR phyosphorylation to similar levels as the low serum condition, 5% serum as compared to 10% normal serum. While rapamycin did not reduce mTOR phosphorylation at this extended time point, this result is consistent with reports of differential effects of rapamycin with

extended treatment and some reports of cell type dependent effects [235, 236]. These results identify the mTOR signaling pathway as a target of indole.

5.8 Summary

Indole , has previously been shown to increase expression of the prototypical AhR target gene CYP1A1 in a concentration-dependent manner and also antagonize TCDD mediated induction of CYP1A1 [72]. However, based the finding that indole attenuated LPS induction of TNF-α accumulation in BMDMs, the I3-BSA conjugate was synthesized to elucidate whether a membrane impermeable indole-containing compound retained function in macrophages. The I3-BSA conjugate attenuates LPS induction of TNF-α in macrophages significantly more at a much lower concentration (90% inhibition at 100 µM) than indole alone (30% at 1 mM) (**Figure 15**). The effect of I3-BSA treatment on LPS induction of TNF-α accumulation was not altered or reduced by phagocytosis inhibitors, nor was I3-GFP detected within macrophages (**Figure 16**), suggesting that the conjugates were not crossing the cell membrane either by phagocytosis or another means.

A



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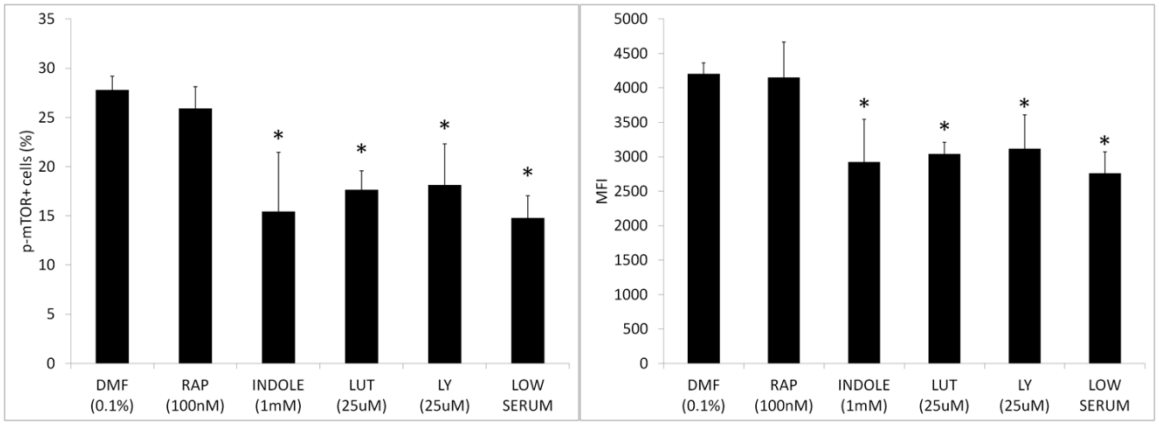

Figure 18. Effect of indole on macrophage p-mTOR as measured by ICS-FACS. Cells were treated with the compound (indole, RAP, LUT, LY) for 22 h. A) FACS plots showing CD11b (APC-Cy7) positive BMDMs costained for p-mTOR (Alexa488). B) Data shown are from three biological replicates of BMDMs per experiment obtained from two independent isolations. "*": statistical significance between treated and solvent control using the Student's t-test at $p < 0.05$.

The activity of the I3-BSA molecule suggested indole functioned through some cell surface receptor. The SLC7A5 receptor was identified as a potential cell surface interacting protein with indole based on the structural similarity of indole to other aromatic amino acid binding ligands of SLC7A5 (e.g. tryptophan). Interestingly, inhibition of SLC7A5 function with the chemical inhibitor BCH augmented indole attenuation of TNF-α accumulation in macrophages. Indeed indole was shown to reduce the phosphorylation of mTOR (**Figure 18**), a crucial metabolic signaling protein which is involved in amino acid uptake and is part of a feedback loop in SLC7A5 expression based on amino acid availability. These results suggest indole functions beyond attenuating inflammation in macrophages and indicate that indole may alter the metabolic state of these cells as well.

6. THE EFFECT OF INDOLE ON MACROPHAGE POLARIZATION

6.1 Introduction

The macrophage has been traditionally described as a phagocytic cell responsible for performing homeostatic maintenance and defense against pathogen infection. Moreover, macrophages have distinct roles in antigen presentation, the development and persistence of inflammation, persistence of tumors and the development of obesity related insulin resistance [20, 32, 33, 35, 153, 237]. Due to the differing microenvironments local macrophages experience *in vivo*, resident tissue macrophages are phenotypically distinct from the dogmatic M1 and M2 phenotypes. The M1 and M2 notation was established as an analogous nomenclature to the TH1 and TH2 phenotypes for CD4+ T-helper cells [34, 37]. Macrophage polarization therefore encompasses more than a single cell surface marker and has expanded into a more complete phenotypic and metabolic characterization of the cell state including metabolic product and functional cytokines profiles [36-39, 158, 238, 239]. Indeed, the field is beginning to realize that the end state of *in vitro* phenotypic polarization of macrophages depends not only on the culture conditions used to differentiate the progenitor cells, but also the source and state of the progenitors [154, 240, 241]. Moreover, the accurate description of the macrophage *in vivo* is poorly described by a single cell surface marker and instead requires much more functional information to accurately determine the role of the tissue specific macrophage as it fills its role in the host.

6.2 Indole alters macrophage polarization

Since indole reduced LPS stimulation of TNF-α production in a macrophage cell line as well as in BMDMs, the effect of indole treatment on polarization of BMDMs was investigated. Mature BMDM's initially considered to be in an unpolarized or M0 state identified as $F4/80^{(+)}CD11b^{(+)}CD11c^{(-)}/CD206^{(-)}$ (**Figure 19**) were treated with 250 ng/mL of LPS to induce polarization to an activated (M1) state that is characterized by expression of the CD11c surface marker. Similarly, unpolarized M0 macrophages were polarized to an alternatively activated (M2) state and CD206 expression with either 20 ng/mL IL-4 or 20 ng/mL IL-10 for 48 hr.

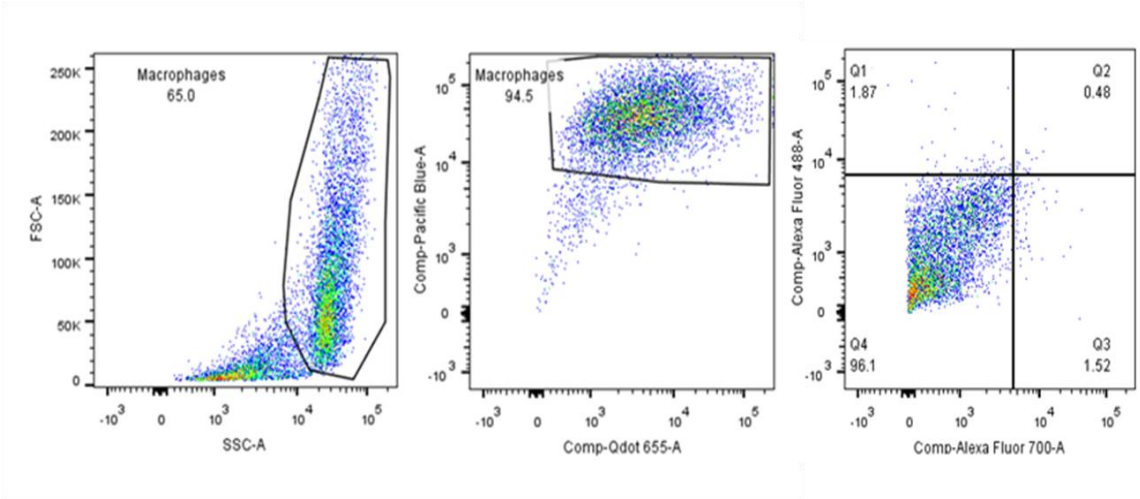

Figure 19. BMDM scatter plots showing cell gating strategy. Cells were gated out from debris using SSC x FSC. Cells stained positive for CD11b (Pacific Blue) and F4/80 (Qdot) were considered mature macrophages for analysis. F4/80$^{(+)}$CD11b$^{(+)}$CD206$^{(+)}$ (Alexa 488) were considered M2 positive and F4/80$^{(+)}$CD11b$^{(+)}$CD11c$^{(+)}$ (Alexa 700) were considered M1 positive.

Indole did not significantly decrease LPS dependent M1 polarization as shown by the similar % of $CD11c^{(+)}/CD206^{(-)}$ subset of cells from the $F4/80^{(+)}CD11b^{(+)}$ population (**Figure 20B**). This was noteworthy as our data also show that TNF- α is characteristically produced by inflammatory M1 polarized macrophages [37, 39] and indole reduces TNF-α production in BMDMs stimulated with LPS (**Figure 20A).** These results were independent of whether BMDMs were grown with or without macrophage colony stimulating factor (M-CSF). One possible explanation for this phenomenon is since M1 macrophage polarization and CD11c expression requires extended incubation (48 h) with the polarization signal, it is possible that indole may not be able to attenuate sustained LPS-mediated signaling and inflammation. Alternatively, it is possible that indole reduces TNF-α production through a TNF-α specific pathway at early time points without significantly affecting the macrophage polarization machinery.

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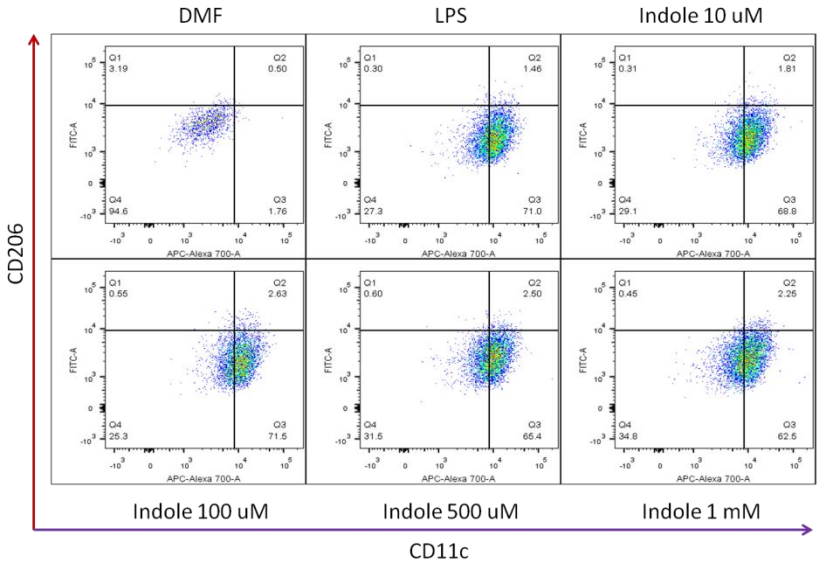

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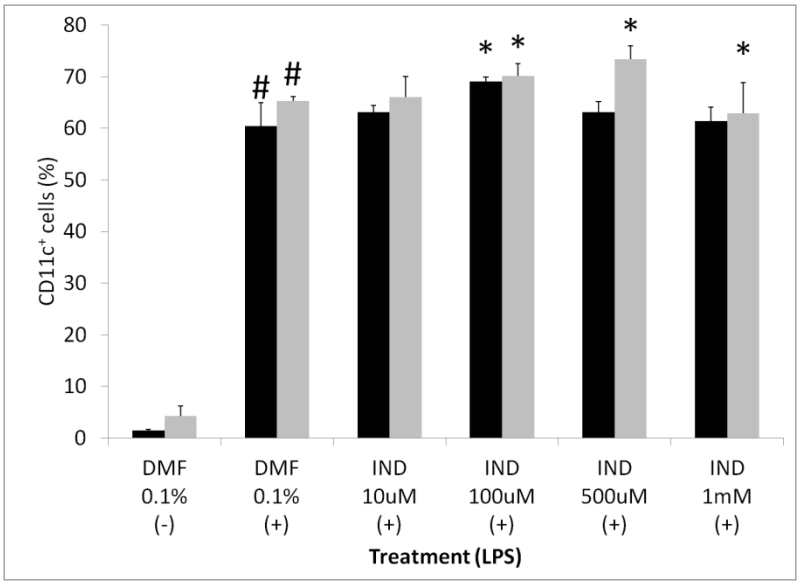
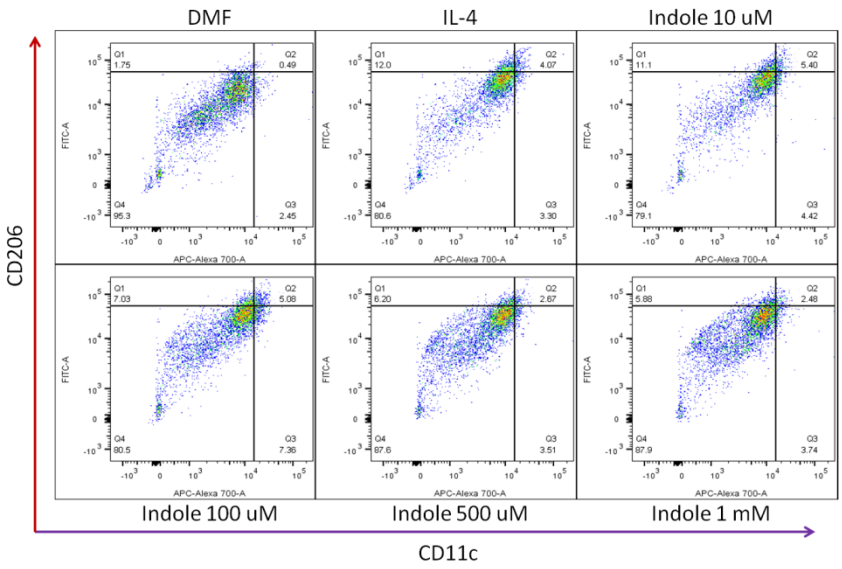


Figure 20. ICS-FACS analysis of macrophage polarization. A) BMDM scatter plots. B) Stimulation with 250 ng/mL LPS with (gray bars) or without (black bars) M-CSF for 48 h increased the percentage of $CD11c^{(+)}$ cells while indole decreased the percentage of $CD11c^{(+)}$ cells in a concentration dependent manner up to 1 mM indole. "-/+" indicates absence or presence of LPS. Data shown are from three biological replicates of BMDMs per experiment obtained from two independent isolations. "#": statistical significance between stimulated and unstimulated control or "*" between indole-treated and solvent stimulated using the Student's t-test at $p < 0.05$.

To extend the investigation of indole's effect on M2 polarization, BMDM's stimulated with either 20 ng/mL of IL-4 or 20 ng/mL of IL-10 for 48 h in the presence of indole at concentrations between 10 μm and 1 mM. Surprisingly, indole treatment attenuated both IL-4 and IL-10 dependent polarization of M0 macrophages to M2 macrophages. Indole treatment reduced the population of $CD206^{(+)}$ BMDM's in a dose dependent manner for both IL-4 treated (**Figure 21A**) cells by 25% at 1 mM and IL-10 treated (**Figure 21B**) cells by 20% at 500 μM.

It is interesting that indole attenuates both IL-4 and IL-10 induction of the M2 macrophage polarization marker CD206 in BMDMs without significantly affecting LPS induction of the M1 cell surface polarization marker CD11c. The attenuation of M2 polarization, regardless of the cytokine signal used implies indole functions through a conserved mechanism utilized by both IL-4 signaling and IL-10 signaling. The mTOR pathways has well established roles in macrophage polarization [55, 229].

A



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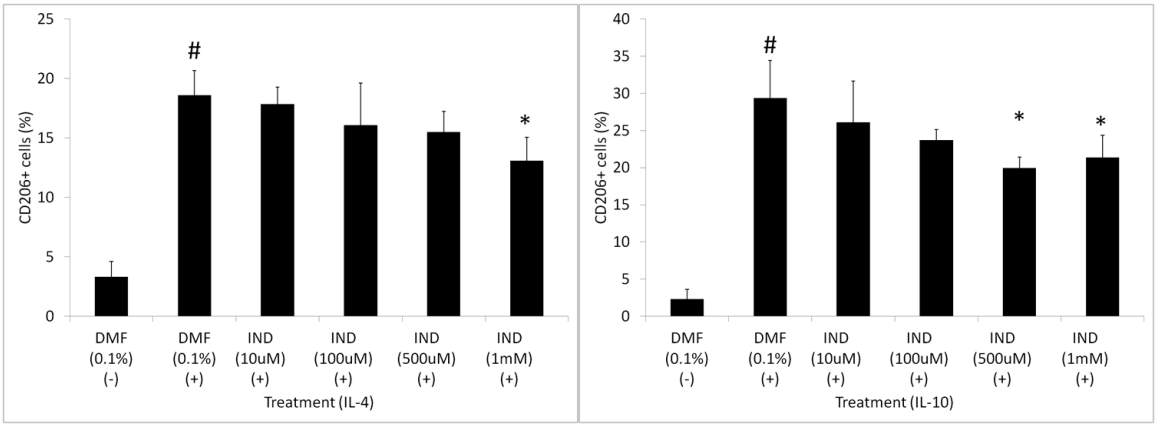

Figure 21. FACS analysis of macrophage polarization. A) BMDM scatter plots. B) BMDMs were stimulated with 20 ng/mL IL-4 or 20 ng/mLIL-10. IL-4 and IL-10 increased the percentage of cells $CD11c^{(-)}/CD206^{(+)}$. Indole treatment decreased the percentage of CD206(+) cells up to 1 mM indole. "-/+" indicates absence or presence of IL-4 or IL-10. Data shown are from three biological replicates of BMDMs per experiment obtained from two independent isolations. "#": statistical significance between stimulated and unstimulated control or "*" between indole-treated and solvent stimulated using the Student's t-test at $p < 0.05$.

6.3 Macrophage polarization gene expression

Indole altered short duration LPS induction of TNF-α in BMDMs without significantly altering the typical marker of BMDM polarization, CD11c cell surface expression. In addition, indole reduced the IL-4 and IL-10 dependent induction of the M2 cell surface marker CD206 at the highest concentration tested (1 mM). To further understand the effect of indole on macrophage activation, the effect of indole on stimulant induction of genes known to be induced in polarized macrophages was interrogated. BMDM's were pre-treated with indole for 4 hours then stimulated with either 250 ng/mL LPS to induce M1 polarization gene expression or 20 ng/mL IL-4 to induce M2 polarization gene expression for an additional 24 or 48 hours in the continued presence of indole. At the end of the incubation period, gene expression was measured by qRT-PCR.

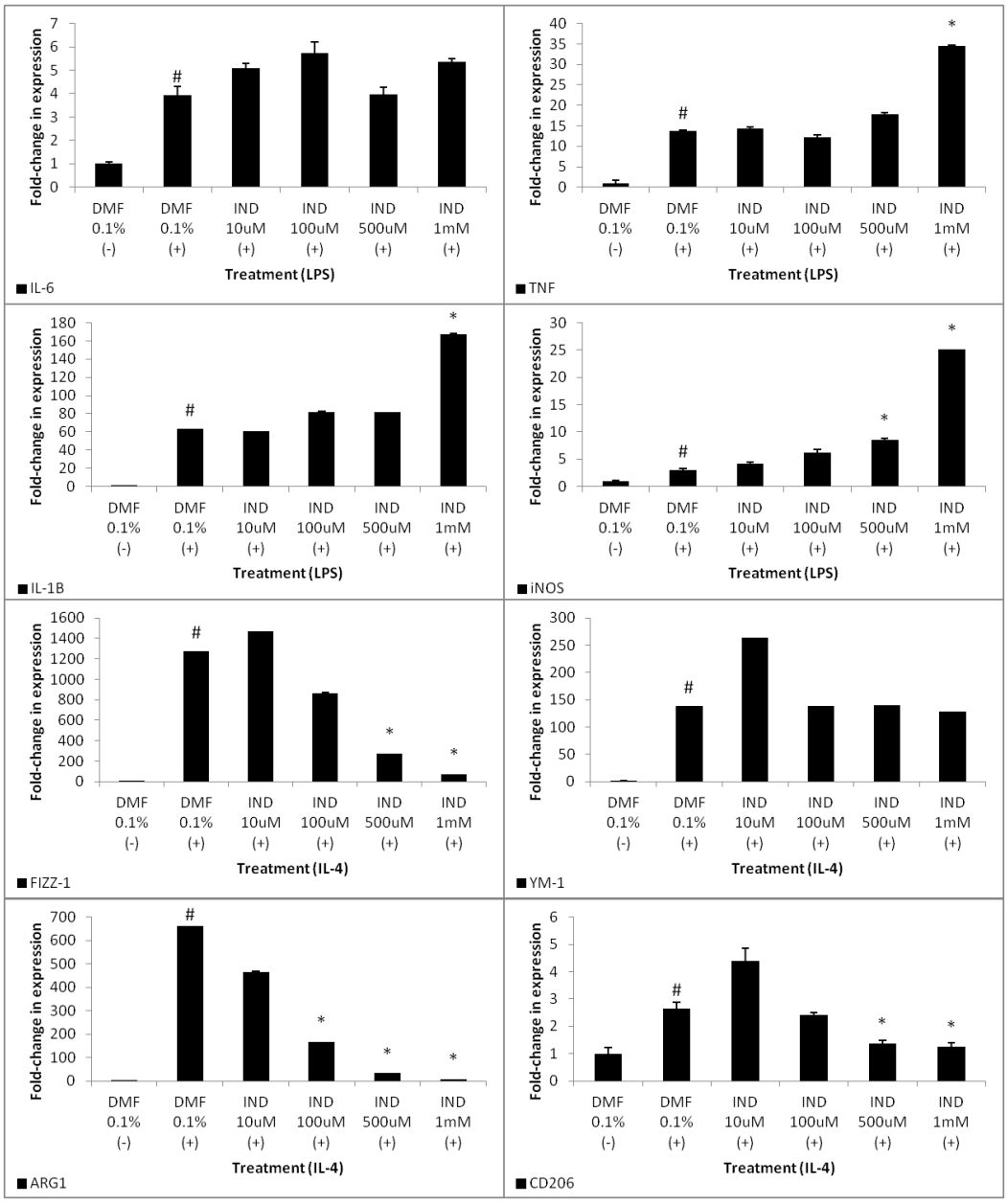

Figure 22. qRT-PCR gene analysis of macrophage polarization. BMDMs were stimulated with 250 ng/mL LPS (M1) or 20 ng/mL IL-4 (M2) for 24 h. "-/+" indicates absence or presence of LPS or IL-4. Data shown are from three biological replicates of BMDMs per experiment obtained from two independent isolations. "#": statistical significance between stimulated and unstimulated control or "*" between indole-treated and solvent stimulated using the Student's t-test at $p < 0.05$.

Despite previous experiments showing that indole attenuated LPS induction of TNF-α production measured by ICS-FACS, indole did not significantly attenuate LPS induction of the four M1 polarization markers tested (IL-6, TNF-α, IL-1β, and iNOS) at the gene expression level after 24 h stimulation (**Figure 22**). However, after 48 h stimulation with LPS, indole significantly attenuated LPS induction of IL-6, IL-1b and TNF-α at 100 μM and 500 μM indole (**Figure 23**). However, 1 mM indole's effects after this extended incubation were opposite the effects at lower concentrations, as indole actually enhanced the LPS induction of the tested genes (**Figure 23**). The divergent functions of indole at different concentrations could be potentially due to the fact that indole alters multiple pathways (i.e., the mTOR and AhR pathways) in cells and reflect differential activation at the different concentrations tested.

However, indole attenuated IL-4 induction of M2 polarization markers at both 24 h (**Figure 22**) and 48 h (**Figure 23**) time points. Indole's effects on IL-4 induction of FIZZ-1, Arg-1 and CD206 were dose dependent at both time points. Indole also dose dependently attenuated IL-4 induction of YM-1 at the 48 h time point. Indeed, FIZZ-1, YM-1 and Arg-1 gene expression was dramatically induced by IL-4 treatment and 1 mM indole treatment reduced FIZZ-1 and Arg-1 induction in excess of 90% at both 24 h and 48 h time points. These results clearly support previous results showing that indole attenuates IL-4 induction of macrophage M2 cell surface markers.

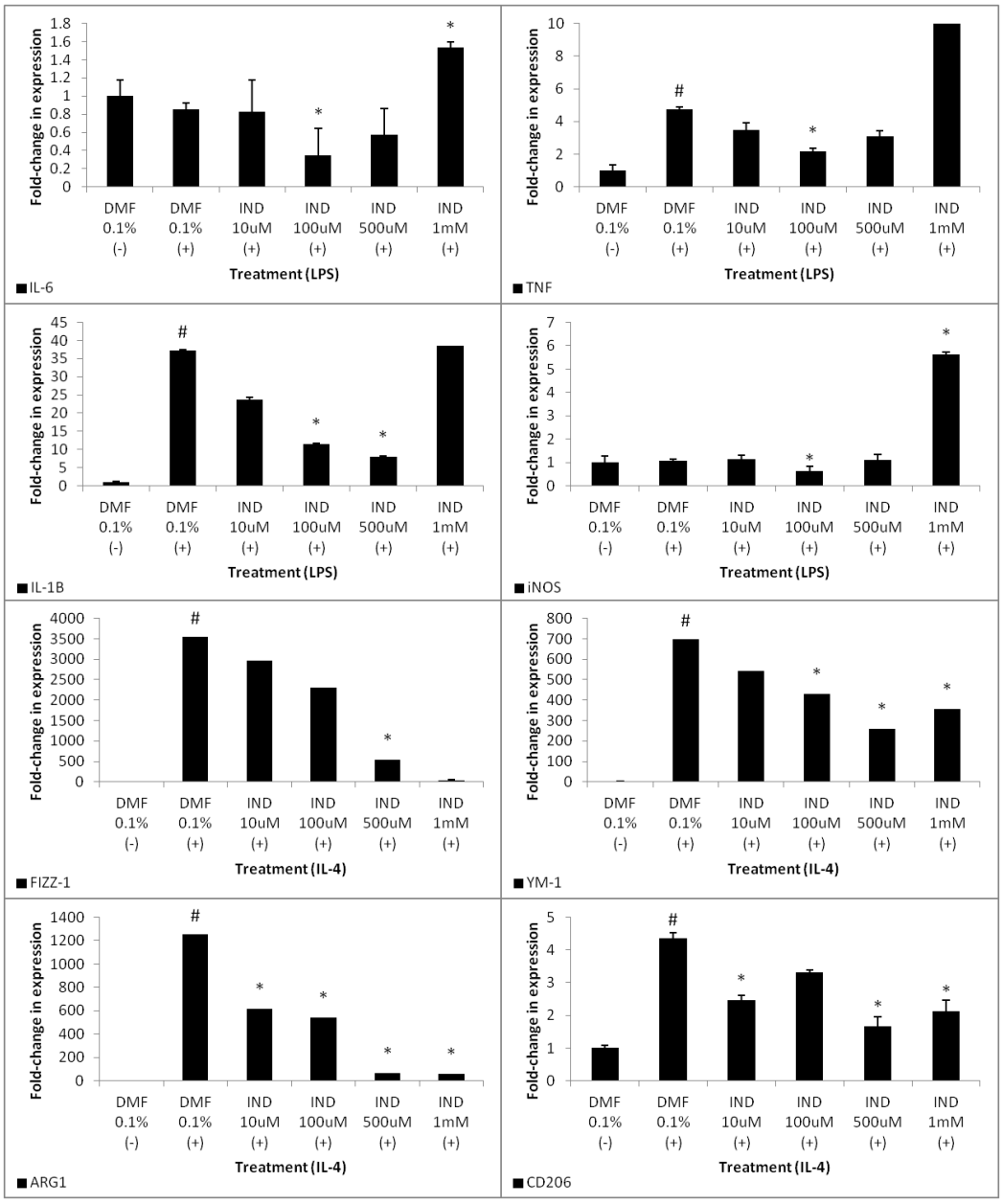

Figure 23. qRT-PCR gene analysis of macrophage polarization. BMDMs were stimulated with 250 ng/mL mL LPS (M1) or 20 ng/mL IL-4 (M2) for 48 h. "-/+" indicates absence or presence of LPS or IL-4. Data shown are from three biological replicates of BMDMs per experiment obtained from two independent isolations. "#": statistical significance between stimulated and unstimulated control or "*" between indole-treated and solvent stimulated using the Student's t-test at $p < 0.05$.

6.4 Macrophage metabolism

In addition to specific cell surface receptor expression, cytokine production and gene expression patterns, polarized macrophages undergo dramatic changes in metabolic function. Classic M1 polarized macrophages demonstrate increased glucose uptake due to an increase in glucose metabolism as the primary source of cellular energy requirements. However, M2 polarized macrophages have a reduced glucose uptake and strongly favor oxidative metabolism. To determine whether indole affected macrophage metabolism, BMDM's were treated with indole for 4 hours then incubated with the fluorescent glucose analog 2-NBDG for an addition 2 hours in the continued presence of indole. Indole significantly cellular uptake of fluorescent 2-NBDG which indicates indole reduces rate of macrophage glucose uptake with a maximum reduction of 35% at 1 mM indole (**Figure 24**). These results show indole reduces the ability of the macrophage to uptake glucose, consistent with the effect of indole on reducing the LPS induction of TNF-α in BMDMs. However, it is unclear if the effect of indole on glucose uptake is compensated for by the cell during polarization as indole did not alter M1 macrophage polarization markers when BMDMs are treated with LPS.

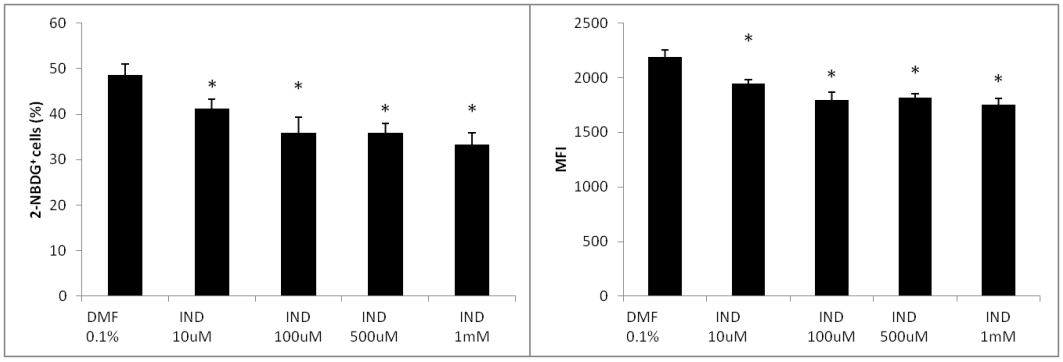

Figure 24. Glucose uptake in BMDMs. Indole reduced 2-NBDG uptake in a concentration dependent manner up to 1 mM indole. Data shown are from three biological replicates of BMDMs per experiment obtained from two independent isolations. "*": statistical significance between indole-treated and solvent treated using the Student's t-test at $p < 0.05$.

Arginine metabolism is also altered in macrophages polarized to an M1 or M2 state, as M1 and M2 polarized macrophages utilize completely different enzymatic pathways to produce different end products from arginine. M1 polarized macrophages utilize arginine to produce the antimicrobial nitric oxide free radical whereas M2 polarized macrophages utilize arginine to produce ornithine that promotes wound healing and tissue repair [169, 242-244]. To assess whether indole altered arginine metabolism in polarized macrophages, BMDMs were polarized to an M1 state by treatment with 250 ng/mL LPS for 24 h or 48 h. At both 24 h and 48 h, LPS significantly induced nitric oxide concentrations in cell supernatants (**Figure 25**). As expected based on gene expression results obtained for iNOS expression (**Figure 23**), indole treatment at concentrations between 10 µM and 500 µM significantly increased the concentration of NO in cell supernatants compared to LPS treatment alone. However, indole treatment at 1 mM caused a moderate decrease in NO concentration in cell supernatants as compared with LPS treatment alone which was not consistent with results obtained measuring iNOS levels by gene expression after 24 h incubation. It is unclear why indole treatment at this higher concentration caused a weak reduction in the metabolic product (NO) while also increasing the expression of the enzyme (iNOS) responsible for the production of NO.

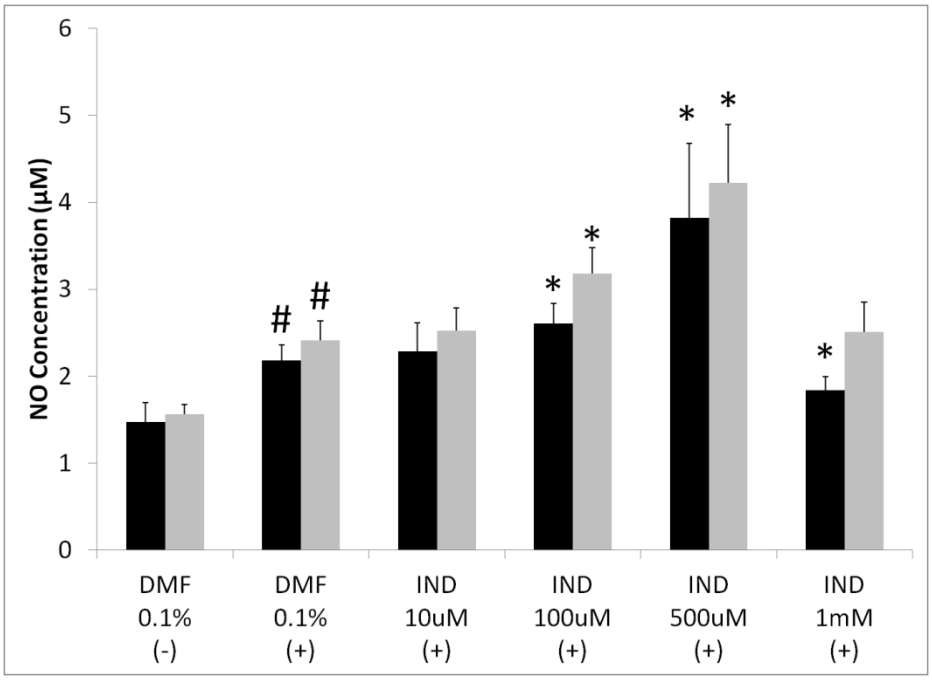

Figure 25. Cell supernatant NO concentration. BMDMs were stimulated with 250 ng/mL mL LPS for 24 h (black bars) or 48 h (gray bars). "-/+" indicates absence or presence of LPS. Data shown are from three biological replicates of BMDMs per experiment obtained from two independent isolations. "#": statistical significance between stimulated and unstimulated control or "*" between indole-treated and solvent stimulated using the Student's t-test at $p < 0.05$.

BMDMs were stimulated to an M2 polarized state by treatment with 20 ng/mL IL-4 for 24 h or 48 h. However, compared to untreated cells, IL-4 did not significantly increase cell supernatant ornithine concentrations as measured by MRM-LC-MS (**Figure 26**) despite ornithine concentrations in cell supernatants being below the limit of detection at 6 h post stimulation which rules out the possibility of ornithine being present in the basal growth media. Therefore we concluded indole treatment did not alter macrophage ornithine accumulation in cell supernatants at either 24 or 48 h post stimulation. However, ornithine concentrations in cell supernatants of IL-4 treated cells may be significantly different in the time between 6 h and 24 h. It may be possible that the time points used in this experiment were insufficient to sufficiently probe ornithine production in response to IL-4 stimulation.

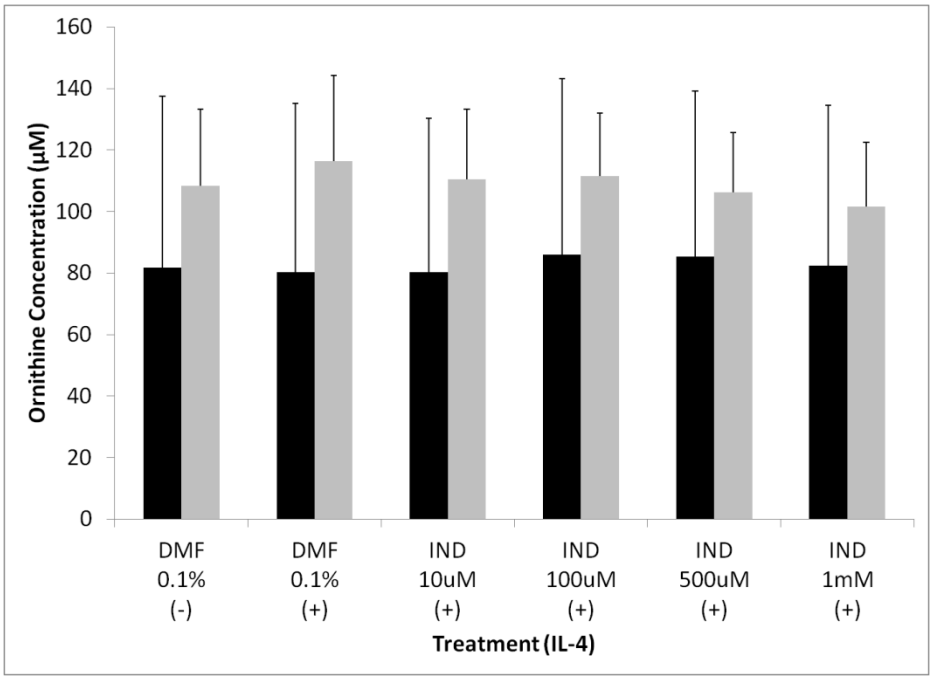

Figure 26. Cell supernatant Ornithine concentration. BMDMs were stimulated with 40 ng/mL mL IL-4 for 24 h (black bars) or 48 h (gray bars). "-/+" indicates absence or presence of IL-4. Data shown are from three biological replicates of BMDMs per experiment obtained from two independent isolations. "#": statistical significance between stimulated and unstimulated control or "*" between indole-treated and solvent stimulated using the Student's t-test at $p < 0.05$.

7. DISCUSSION AND SUMMARY

The presence of a functional, healthy microbiota is essential for the development several host functions and homeostasis [116, 245, 246]. While the identity and abundance of gut bacteria remain to be fully elucidated at the level of individual species, recent advances in metagenomics have begun to establish the phylogenies of microbes in the human intestine [247]. Though interactions in the gut play a significant role in the host (e.g., immune system development during dendritic cell sampling) [3, 248], perhaps more importantly the microbiota produces an extensive array of molecules, many of which are beneficial and putatively regulate homeostasis in the host [60, 249]. This dissertation seeks to investigate the role of one such microbiota-produced metabolite, indole, on the modulation of inflammatory signaling in macrophages and adipocytes.

7.1 The effect of indole on macrophage and adipocyte inflammation

Several studies have described compositional changes in the microbiota in various disease models [250]; however, the differences in the levels of microbiota derived metabolites in normal and disease states and their role in exacerbation or attenuation of disease are poorly understood [59]. In this work, we studied the role of indole on different indicators of inflammation in macrophages and adipocytes. Since indole has been shown to be present in colonic contents at high concentrations [64, 225] and indole-derived metabolites have been identified in circulation [226], we reasoned that indole is

also likely to be present at low levels in murine serum. Using LC-MS we identified indole as being present in murine serum at a concentration of approximately 10 μM, which indicates that indole does reach organs distal from the GI tract. Based on previous results from our lab showing indole attenuates NF-κB activation in response to inflammatory stimuli [65], we hypothesized that indole attenuates inflammation in macrophages and adipocytes. This hypothesis was tested using an *in vitro* model that mimics low grade endotoxemia associated inflammation.

Chronic inflammation in adipocytes and macrophages is a critical contributing factor to chronic inflammation in adipose tissue and the development of insulin resistance and type 2 diabetes. Our data clearly show that exposure to indole attenuates multiple indicators of inflammation in both macrophages and adipocytes. In macrophages, exposure to indole reduced the LPS induced expression of TNF-α and migration of macrophages towards both MCP-1 and ADSM, whereas in adipocytes, indole decreased LPS induced gene expression IL-6 and MCP-1 (Figure X). The role of cytokines such as MCP-1 promoting increased macrophage migration into the adipose tissue and IL-6 impairing insulin signaling in the liver and adipose tissue in chronic obesity associated inflammation have been well established [251]. The notion that a molecule produced by the microbiota modulates different facets of inflammation in response to LPS in macrophages and adipocytes is interesting as the response to LPS is an early event in the establishment of chronic inflammation associated with obesity [19]. Further indole attenuates the expression of IL-6 in adipocytes in response to LPS stimulation (**Figure 8**)

demonstrating indole reduces multiple different inflammatory signaling responses associated with the progression of obesity and insulin resistance.

Paracrine signaling between adipocytes and macrophages is critical for maintaining elevated levels of inflammatory indicators in the adipose tissue in obese subjects [252]. For example, LPS induces the production of chemokines in adipocytes, which leads to infiltration of macrophages in to the adipose tissue. This increase in the population of resident adipose tissue macrophages has been proposed to underlie the sustained inflammation in adipose tissue [150, 253] as the newly resident activated macrophages produce inflammatory cytokines in response to endotoxin and cytokine stimulation. These cytokines in turn stimulate adipocytes to produce pro-inflammatory chemokines and reduce the production of anti-inflammatory adipokines such as leptin and ACDC [148, 254]. The inhibition of macrophage migration to MCP-1 by indole is significant because it suggests that microbiota metabolites such as indole can not only attenuate expression of inflammatory cytokines but also prevent the recruitment of macrophages to adipose tissue.

In addition to modulating LPS-induced inflammation in adipocytes and macrophages, indole also attenuated MSM induced inflammation in adipocytes and ADSM induced migration in macrophages (**Figure 9**). These results suggest that indole attenuates the effects of two main inflammatory triggers associated with obesity, LPS from the microbiota and adipose tissue derived cytokine paracrine signaling between adipocytes

and macrophages [7, 19]. While indole was effective in reducing the response to MSM, it did not attenuate the adipocyte response to TNF-α. This observation could be due to the fact that MSM contains both pro and anti-inflammatory cytokines, and while indole does not attenuate TNF-α signaling, it may modulate other cytokine signaling pathways, which results in the overall reduced inflammatory response observed with MSM. Our data also demonstrates that the microbiota metabolite indole inhibits several facets of adipose tissue inflammation, all of which have previously been shown to be involved in the development of insulin resistance [227]. Since obesity is also characterized by dysbiosis of the microbiota [255], it is intriguing to speculate that the microbiota, through the metabolites it produces, plays an important role in preventing the onset of LPS-mediated inflammation in adipose tissue. It is also interesting to note that indole has been shown to increase tight junction resistance and mucin production both *in vitro* [65] and *in vitro* [64], which may minimize the migration of bacterial endotoxins into circulation. Thus, microbiota metabolites may prevent the inflammatory response to LPS through both minimizing the transport of LPS into circulation as well as by attenuating indicators of inflammation in different host sites.

The molecular mechanisms underlying the effect of indole and other microbiota metabolites in host cells have not been fully elucidated. Recent work from our lab demonstrated that indole is an agonist for the AhR and induces the expression of CYP1A1 gene expression in CaCo-2 intestinal epithelial cells [72]. However, indole also antagonizes activation of AhR signaling by TCDD (as seen by the decrease in CYP1A1

expression). Thus, the role of indole-mediated activation of the AhR in intestinal epithelial cells is not clear. In our study, we observed that indole attenuated the migration of BMDM's derived from $AhR^{-/-}$ mice towards MCP-1 similar to WT mice. This suggests that the effects of indole in macrophages are not mediated through the AhR and additional signaling pathway(s) are involved in mediating the observed anti-inflammatory response elicited by indole. This hypothesis is also supported by the fact that AhR expression has been documented to be reduced or absent in differentiated adipocytes [192]; yet, we observed marked effects with indole on mature differentiated adipocytes (**Figure 8 and 9**).

Our results suggest a model (**Figure 27**) where the microbiota, through anti-inflammatory metabolites such as indole that it produces, act as a "brake" against the onset of inflammation in adipose tissue by minimizing the presence of LPS in circulation and by modulating various indicators of inflammation in macrophages and adipocytes. When the microbiota composition is altered, as in in obesity, this dynamic equilibrium between indole and LPS-mediated inflammation is perturbed as LPS enters circulation due to leakiness in the barrier function of intestinal epithelial cells. In addition, the changes in the microbiota community also lead to a decrease in the production of anti-inflammatory metabolites such as indole, which in turn leads to the initiation of LPS-mediated inflammation in adipose tissue (**Figure 27**). This model is supported by the fact that indole is detected in serum from mice raised on a normal (low-fat) diet at a concentration of ~ 10 μM while it is reduced in mice raised on a high-fat diet. Taken

together, our data suggest an important role for microbiota metabolites in the maintenance of homeostasis in the GI tract.

7.2 Investigation of indole signaling in macrophages

The observation that AhR signaling was not required for indole's anti-inflammatory effect in macrophages is intriguing as it raises multiple questions on the underlying mechanisms. It is possible that the role of the AhR could be tissue or cell context-dependent. That is, the relative abundance of AhR in cell types, such as CaCo-2 human colon cancer cells and MDA-MB-468 and MDA-MB-231 breast cancer cells [72], where indole modulated AhR-dependent gene expression, compared to that in macrophages where AhR expression is lower [128], could determine if the AhR is involved in mediating indole's effects [72, 256]. In addition, the relative affinity of indole to the AhR or other putative receptor(s) in these cell types could also determine the receptor to which indole binds. Indeed, the transcriptional profile of AhR induced genes in macrophages is quite different compared to hepatocytes or breast cancer cells [257, 258], and the lack of AhR engagement in macrophages could simply reflect a difference in possible cell signaling pathways in the different cell types.

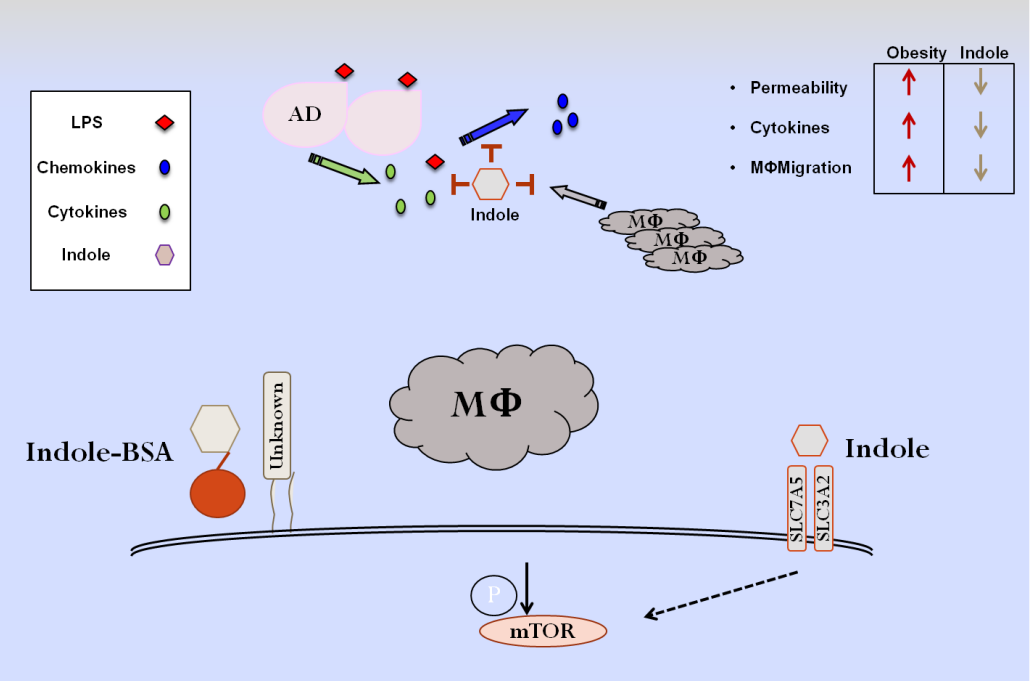

Figure 27. Proposed model describing attenuation of adipose tissue inflammation by microbiota metabolites such as indole. Indole increases tight junction resistance and barrier function to minimize the migration of bacterial endotoxins and LPS into circulation [19]. Indole attenuates LPS-mediated induction of inflammatory cytokine and chemokine production in adipocytes and macrophages. In macrophages, indole acts independent of the AhR and alters the mTOR signaling pathway while I3BSA functions at the cell surface.

Based on the structural similarity between indole and the amino acid tryptophan from which indole is derived (**Figure 1**), we hypothesized that the amino acid transporter SLC7A5 could be a potential cell surface receptor for indole. Therefore, we hypothesized that inhibition of SLC7A5 activity would decrease the transport of indole into the cell and hence decrease the attenuation of TNF-α upon LPS stimulation. Therefore, it was surprising to observe that chemical inhibition of SLC7A5 augmented the attenuating effect of indole on the LPS induction of TNF-α in macrophages. While it is possible that indole can still enter the cell through a different transporter, this observation suggested that indole does not need to be internalized through SLC7A5 in macrophages for it to modulate inflammation.

The surprising increase in attenuation of LPS-mediated inflammation in macrophages led to the hypothesis that indole binds to a cell surface receptor in macrophages. Based on previous reports identifying a cell surface receptor for the estrogen receptor using an estrogen-BSA protein conjugate [201, 209], we designed a conjugate of indole-3-carboxaldehyde with BSA (I3-BSA) conjugate that would not freely cross the cell membrane and hence, can be localized to the cell surface. This would allow us to test the hypothesis that indole does not need to be internalized for it to be attenuate LPS-mediated inflammation in macrophages. The fact that the I3-BSA conjugate attenuated LPS induction of TNF-α in both RAW264.7 macrophages and BMDMs to a much greater extent than either the precursor I3CA or indole (**Figure 15**), and that this activity did not require active phagocytosis or acidification of the endosome (**Figure 15**)

strongly suggests that the I3-BSA conjugate need not enter the cell to exert its anti-inflammatory effect.

While our studies to demonstrate localization of indole to the cell surface using an I3-GFP conjugate were not successful, we also found no evidence of I3-GFP within the cell (**Figure 16**) after 16 h incubation. This observation suggests that the I3-GFP conjugate does not enter the cell which is consistent with the results obtained with I3-BSA. The our data with $AhR^{(-/-)}$ BMDMs showing inhibition of TNF-α expression by indole upon LPS stimulation, and the increased inhibition observed with the I3-BSA relative to both indole and indole-3-carboxaldehyde, as well as the increased attenuation when SLC7A5 is inhibited compared to the control, all support the hypothesis that indole's effect on inhibition of LPS-mediated inflammation in macrophages is independent of the AhR and is likely mediated through a cell surface receptor.

Increased mTOR signaling has been implicated in amino acid-induced insulin resistance progressing from obesity and type 2 diabetes through the downstream target of mTOR, S6K. A transcriptional feedback mechanism between amino acid transport through SLC7A5 and mTOR pathway activation has been previously described [177] in which a decrease in the intracellular amino acid pools leads to down-regulation of mTOR activity. This, in turn, increases SLC7A5 expression and import of amino acids into the cell, which reactivates mTOR activity and down-regulates SLC7A5. Results obtained with the SLC7A5 inhibitor BCH in which both indole and I3CA reduced TNF-α

expression (**Figure 18**), indole and I3CA both significantly reduced mTOR phosphorylation. These results suggest that the reduction in mTOR phosphorylation also caused a reduction in mTOR pathway activity. The fact that the same response (i.e., decrease in mTOR phosphorylation in BMDMs derived from $AhR^{(-/-)}$ mice with indole and I3CA), demonstrates that this effect is independent of the AhR. The mTOR pathway integrates signals from multiple signaling pathways including amino acids, insulin, leptin, hypoxia and DNA damage [259]. One interaction of particular interest is the role of the mTOR regulated gene hypoxia-inducible factor 1-alpha (HIF-1α) which forms a complex with hypoxia-inducible factor 1-beta (HIF-1β), also known as ARNT [260, 261]. Indole altering HIF-1α expression and therefore interaction with HIF-1 β, could lead to altered availability off ARNT for AhR binding. Activation of the mTOR pathway also leads to transcription of sterol regulatory element-binding protein (SREBP1). In this pathway, HIF-1α stimulates glucose uptake and glycolysis by activating transcription of genes involved in glycolysis (e.g. GLUT1) [262]. SREBP1 then promotes cell proliferation downstream of mTORC1 and S6K1, a direct target of mTOR [263]. Through these mTOR dependent mechanisms, indole could be reducing macrophage M1 and M2 polarization.

7.3 Effects of indole on macrophage polarization

The effects of indole on the LPS induction of M1 macrophage polarization paradoxically contrasted with the effects of indole on the LPS induction of TNF-α in BMDMs and

RAW264.7 cells. In the latter model, indole clearly attenuated LPS induction of cytokine production which led us to hypothesize that indole would also inhibit other markers of M1 macrophages. However, in BMDMs polarized to an M1 state, indole significantly enhanced LPS induced iNOS gene expression and NO production. Indole also had no significant effect on LPS induction of the classic M1 macrophage cell surface receptor CD11c. Furthermore, indole displayed time dependent effects on gene expression with moderate enhancement of LPS induction of cytokine gene expression 24 hours post LPS stimulation and strong attenuation of LPS induction of IL-6 and IL-1β expression 48 hours post stimulation. Indole also reduced glucose uptake in BMDMs indicating that indole treatment reduces the metabolic ability of the cell to bias towards an M1 polarized macrophage. Taken together, indole appears to selectively attenuate LPS induction of M1 associated cytokines while augmenting the expression of the antimicrobial product NO as well as the expression of iNOS.

In stark contrast, polarization to the M2 state was strongly and consistently attenuated by the presence of indole with the exception of ornithine production. However, unlike LPS induction of the M1 macrophage polarization marker CD11c, indole strongly attenuated IL-4 and IL-10 dependent induction of CD206, a cell surface receptor used to identify M2 polarized macrophages. Further, indole significantly reduced IL-4 induction of FIZZ-1, YM-1, Arg-1 and CD206 gene expression in BMDMs. IL-4 induction of M2 polarization involves the phosphorylation and activation of Akt eventually leading to the activation of the transcription of M2 related genes [55]. Since indole treatment reduces

Akt phosphorylation, and Akt signaling has been shown to be important for M2 macrophage polarization, it is possible that indole directly attenuates IL-4 induction of M2 polarization. Alternatively, indole could attenuate M2 macrophage polarization by reducing mTOR activity as mTOR also promotes M2 macrophage polarization [55]. However, these potential mechanisms of action need not be mutually exclusive as the Akt and mTOR pathways are interconnected. Similar to the effect of indole, the mTOR inhibitor Rapamycin attenuates IL-4 induction of M2 macrophage gene expression which is consistent with the hypothesis that the effects of indole are mediated through mTOR [55]. While our data clearly show that indole attenuates macrophage polarization towards an M2 state, the mechanism(s) involved in this are not fully understood.

Metabolically, indole treatment reduces glucose uptake in BMDMs and reduces mTOR activation. The mTOR pathway has been identified to integrate several signaling pathways in both the LPS induced M1 macrophage state as well as the IL-4 induced M2 macrophage state [55]. Indeed, both LPS stimulation and IL-4 stimulation induce an increase in mTOR activity in BMDMs [55]. Mechanistically, it is clear that indole alters LPS inflammatory signaling in macrophages independent of the AhR. Indole may interfere with LPS induction of TNF-α and M1 polarization, by reducing mTOR phosphorylation and activity. Indole attenuates LPS induction of macrophage the production of TNF-α and M1 polarization. LPS stimulation of TNF-α is an NF-κB dependent process, and indole has previously been shown to attenuate TNF- α induction of NF-κB activation in HCT-8 enterocytes [65]. Additionally, indole has been identified

as an agonist-antagonist of the AhR [72]. Therefore, it is not surprising that indole functionally alters so many different signaling events in macrophages as indole alters a key signaling pathway in mTOR which functionally integrates a diverse array of signals. While indole clearly alters multiple signaling pathways, the exact mechanism(s) remains unknown.

7.4 Future directions

The molecular signaling events associated with indole treatment in this work and other reports encompasses an incredibly diverse array of pathways and targets [64, 65, 72, 264]. The mTOR pathway serves to integrate both intracellular and extracellular signals and functions as a central regulatory pathway with roles in cell metabolism, growth, survival, immunity, adipogenesis and tumor formation [43]. With the discovery of the mTOR pathway as a target of indole, it is no surprise that indole alters such a diverse array of signaling events including those involved in regulating macrophage migration, polarization, inflammatory cytokine production and metabolism. Thus, a more comprehensive investigation of the effect of indole on mTOR signaling needs to be carried out.

Indole drastically reduces macrophage migration towards both adipocyte derived chemokines in the form of ADSM and specifically MCP-1, a chemokine significantly induced in response to LPS treatment of adipocytes. MCP-1 signaling occurs through

binding to the C-C chemokine receptor 2 (Ccr2) which utilizes the Akt signal transduction pathway [265]. It is of interest to determine whether indole attenuated MCP-1 activation of the Akt pathway and if this effect was dependent on mTOR, or if indole signaled through some alternative mechanism. It is also possible that the dramatic effects of indole attenuating BMDM migration are mediated through indole modulation of mTOR.

Since pre-incubation of cells with indole led to a more pronounced effect in attenuating BMDM migration in response to MCP-1 compared to cells not pre-treated with indole, an alternate hypothesis could be that this effect is due to indole altering the cell surface expression of Ccr2 [266]. A reduction in the cell surface expression of Ccr2 would also explain the significant reduction in BMDM migration towards MCP-1. Ccr2 expression is controlled by the AMP-activated protein kinase (AMPK) signaling pathway which also functions as an activator of the TSC1/2 complex in the mTOR pathway [266, 267]. Therefore, another future line of investigation could be to investigate the effect of indole on Ccr2 expression and the role of AMPK signaling in the observed response. Indole potentially reducing Ccr2 expression may provide additional information on how indole attenuates BMDM migration towards the more complex mixture of chemokines contained in ADSM.

I3-BSA attenuates LPS induction of TNF-α in macrophages by 90% compared to untreated cells (75).Yet, despite this strong effect of I3-BSA in comparison to the parent

molecule I3CA, I3-GFP could not be visually detected localized to the cell membrane as would be expected for a strong indole-ligand interaction. Understanding the structure and extent of conjugation of the I3-BSA molecule (i.e., the stoichiometric ratio of indole to BSA molecules) by LC-MS may provide insight on the number of available indole moieties on I3-BSA which could interact with macrophage surface receptors. The effects of the I3-BSA conjugate in reducing LPS stimulation of macrophage TNF-α production also reinforces the idea that the indole moiety is an active component of several other microbiota derived tryptophan metabolites (e.g. indole-3-acetate and indoxyl). The indole class of microbial metabolites needs to be examined to fully understand the importance of tryptophan metabolites in GI homeostasis, and as in the case of indole, homeostasis of the host beyond the GI tract.

Obesity associated chronic inflammation is a critical contributing factor to the development of insulin resistance and type 2 diabetes [7]. As research continues to detail the mechanistic development of events leading to type 2 diabetes, investigations will begin to focus on inhibition of the individual critical inflammatory events in an effort to prevent drastic the increase in morbidity and mortality due to obesity induced insulin resistance and type 2 diabetes. The microbiota provides crucial signals for the development and function of the host and the metabolites and products of these microorganisms are not only necessary for host homeostasis, they also influence host susceptibility to various disease states [77, 92, 268]. Through indole, we show that the microbiota produce functional molecules which have the potential to limit the

development of obesity associated insulin resistance. We further identify indole functions independent of the AhR in BMDM's. As an alternative, we identify indole alters the mTOR signaling pathway in BMDM's. Current efforts are underway to conclusively identify whether the SLC7A5 receptor is involved in indole signaling.

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