EFFECTS OF MICROBIALLY DERIVED TRYPTOPHAN METABOLITES ON IMMUNE CELLS

A Dissertation

by

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ABSTRACT

The gut microbiota has been recognized for influencing human health and immune homeostasis, with metabolites being a biochemical mediator of these functions. The immune-modulatory effects of microbially-derived tryptophan metabolites (MDTMs) have been demonstrated in various immune contexts, we report further characterization of their effects in epithelial cells, macrophages, and T cells. We find the MDTM indole acts as a potent inhibitor of TCDD mediated aryl hydrocarbon receptor (AhR) signaling in colon epithelial cells and as an inhibitor of LPS-stimulated inflammation in macrophages. The effects of indole on macrophages are AhR independent, suggesting cell-type specific signaling mechanisms. Inhibition of phosphatidylinositide-3'OH kinase (PI3K), c-JUN NH2-terminal protein kinase (Jnk), p38 mitogen-activated protein kinase (MAPK), or Akt kinase pathways failed to ablate the reduction of LPS-stimulated TNF accumulation by indole. Interestingly, we fail to capture an indole-mediated reduction in Tnf mRNA levels. Inhibition of proteasomal and lysosomal degradation pathways also fail to ablate indoles effects. The microRNAs miR-181b and miR-124 may act as repressors of TNF translation in macrophages. However, we find no induction of their expression by indole.

To further probe the underlying signaling mechanisms of indole, we synthesize a novel cell-impermeant molecule with an indole moiety covalently bound to bovine serum albumin (I3B), limiting interactions to cell-surface mediated effects. We observe a 50-fold increase in the potency of I3B compared to indole, with 10 μM I3B having a

similar effect on TNF accumulation in macrophages as 500 μM free indole. Transcriptome profiling and subsequent experiments demonstrate I3B functions in part through NRF2.

In T cells, we find indole augments the differentiation of naïve T cells to regulatory T cells (Tregs) and inhibit differentiation to T helper 17 cells (Th17s). Another MDTM, 5-hydroxyindole (5-HI), has opposing effects, inhibiting Treg and augmenting Th17 differentiation. Transcriptome profiling suggests activation of IRF3 and IRF7 in indole-augmented Tregs, while 5-HI augmented Th17s exhibit signatures of increased TNF and LPS mediated signaling pathways with a particularly high level of *Il22* induction.

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1. INTRODUCTION

1.1. Background and Motivation

The human gastrointestinal (GI) tract is colonized by ~10^{13} microorganisms belonging to over 1,000 species, collectively termed the gut microbiota[1,2]. The diversity of microbial genes found in the human microbiome is roughly two orders of magnitude greater than those found in the human genome, illustrating the potential for microbes to contribute in increased functionally in the gut[3]. Indeed, the healthy intestinal microbiota contributes to human health by facilitating efficient energy metabolism, breaking down otherwise indigestible polysaccharides, and contributing to the detoxification of xenobiotic compounds[4]. Non-pathogenic microbes present low levels of exogenous bacterial and fungal signals, training immune cells sampling the intestinal milieu to develop tolerance to innocuous colonizers[5]. Occupation of the GI tract by symbionts also contribute significantly to impeding colonization by pathogenic species[6]. More recently, the intestinal microbiota has also been shown to play a central role in the development and maturation of the immune system[7].

The importance of the microbiota in human health is supported by numerous studies showing that alterations in intestinal microbial composition (dysbiosis) is associated with disease[8-19]. A precise definition of the core (or conserved) microbiota among humans, and what constitutes dysbiosis is lacking; nevertheless, it is well established that exposure to environmental toxicants, dietary perturbations, antibiotics, and lifestyle have all been shown to alter the composition of the microbiota to different

degrees at different taxonomical levels[20-23]. Microbiota dysbiosis is correlated with different diseases such as obesity, diabetes, inflammatory bowel disease, and cancer[9,11,16,17,24]. At the phylum level, increased abundance of *Firmicutes* and decreased abundance of *Bacteroidetes* are associated with Crohn's disease and ulcerative colitis[25]. The same is observed in genetically obese mice, suggesting relative populations of these phyla play a critical role in multiple disease states[26]. While these associations are correlative, recently, causal relationships between microbiota dysbiosis and different diseases such as obesity, type 2 diabetes and insulin response, ulcerative colitis, and depression have also been established[17,27-30]. Interestingly, the diseases associated with microbiota dysbiosis are not restricted to the GI tract alone; cardiovascular diseases, autism disorders, Parkinson's disease, non-alcoholic fatty liver disease (NAFLD), depression, and cognitive disorders have also been linked to dysbiosis[8-19]. As the microbiota has been found to be rapidly altered by environmental and dietary factors, targeting the microbiota and reversing or modulating dysbiosis is emerging as an attractive option for therapeutic development for these conditions[20,21].

While several mechanisms have been proposed to explain the role of the microbiota in the above-mentioned diseases, a common thread that is emerging is that the microbiota is involved in modulating the chronic inflammation that is associated with these diseases. Thus, the intersection of gut microbiota and inflammation presents an avenue for mechanistic investigations of microbiota function.

Seminal studies have associated the presence or absence of a few specific microorganisms and their secreted products to diseases, for example, the association of

Bacteroides Fragilis and its secreted Bacteroides fragilis toxin to colorectal cancer[31,32]. However, a mechanistic understanding of how the microbiota modulates health and disease is lacking. A common hypothesis that has been proposed is that metabolic products produced by the microbiota signal to host cells to modulate their phenotypes and responses. The microbiota is capable of directly synthesizing essential amino acids, directly contributing to host nutrition[4,33]. In addition to amino acids, one of the most studied classes of microbiota-produced metabolites are short-chain fatty acids (SCFAs) that are formed as a result of fermentation of indigestible polysaccharides in the colon[34,35]. Bacterial production of the three most abundant SCFAs, butyrate, acetate, and propionate, is critical to maintaining intestinal health, directly feeding the intestinal epithelium and promoting growth and maintenance[36]. Microbially-derived secondary bile acids are another class of metabolites that are involved in carbohydrate metabolism and promotion of glucose tolerance.

Microbially derived amino acid metabolites are an emerging class of microbiota metabolites that have also been shown to regulate immune homeostasis and tolerance in the GI tract[37,38]. Biosynthesis of microbially-derived tryptophan metabolites (MDTMs) such as indole and indole-like molecules have demonstrated anti-inflammatory or tolerance-promoting phenotypes in various immune cell types relevant to the GI tract (epithelial cells, macrophages, T cells) as well as in promoting epithelial barrier integrity that indirectly modulates intestinal inflammation[39-46]. MDTMs are also detected in circulation and in non-intestinal organs (e.g., liver), providing an additional mechanism for modulating disease at distal sites[47]. Indeed, MDTMs have demonstrated anti-

inflammatory properties in hepatocytes, keratinocytes, and astrocytes[42,44,45,48,49]. Since ~90% of dietary tryptophan is absorbed in the small intestine and metabolized by the host, modulating the biosynthesis of molecules such as kynurenine and the neurotransmitter serotonin are also possible mechanism through which the microbiota may modulate host signaling in organs and tissue other than the GI tract[50,51].

It is important to note that while several microbiota metabolites have been associated with beneficial health effects (i.e., amelioration of disease), some metabolites are also implicated in the etiology of disease onset and progression. For example, the microbiota has been shown to synthesize various trimethylamine precursors and these metabolites have been associated with cardiovascular disease (CVD), atherosclerosis, and thrombotic events[14,15,52-54].

Targeted investigations of microbially-derived metabolites in different immune cell types have yielded detailed information on the mechanisms through which these metabolites exert their either beneficial or detrimental effects. Butyrate can directly upregulate interleukin (IL)-18 production by the colonic epithelium, suppressing colonic inflammation *in vivo*[55]. In the context of T cells, SCFAs have been shown to increase the abundance and homeostatic function of colonic regulatory T cells (Tregs), a critical immune cell in regulating intestinal inflammation[56]. In macrophages, butyrate has been shown to stimulate the differentiation of monocytes into macrophages, while decreasing secretion of pro-inflammatory IL-6, IL-12, and nitric oxide upon lipopolysaccharide (LPS) stimulation. Of the MDTMs, indole is perhaps the most abundant in the human gut, with fecal concentrations being reported in the millimolar range[57-60]. Previous work

in colonic epithelial cell lines have demonstrated that indole augments production of anti-inflammatory IL-10 while downregulating tumor necrosis factor (TNF)-induced expression of pro-inflammatory IL-8, and upregulating expression of genes involved in epithelial barrier function[39,40]. Previous work have also demonstrated other MDTMs, such as indole-3-acetic acid and tryptamine, are capable of modulating macrophage inflammatory responses *in vitro*, inhibiting the induction of *Tnf*, *Il1b*, and *Ccl2*[44]. However, phenotypic characterization of the effects of indole on macrophages and T cells are lacking. One mechanistic pathway that has been identified for MDTMs is signaling through the aryl hydrocarbon receptor (AhR). In epithelial cells, indole and other MDTMs have demonstrated mild AhR agonist activity, and significant antagonistic activity against the synthetic AhR ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)[41,61]. Alternatively, the anti-inflammatory effects of indole-3-acetic acid on macrophages were found to be independent of the AhR, highlighting a potential for cell-context dependent signaling mechanisms and the engagement of additional receptors by MDTMs[46].

1.2. Aims

This work will aim to address the knowledge gaps in the mechanistic understanding of MDTM signaling in immune cells. Using indole as the prototypical MDTM and two immune cell types (T cells and macrophages), we seek to further delineate the involvement of the AhR in indole signaling, and to identify additional signaling pathways that may be involved in facilitating immune regulation by MDTMs

in a cell-context dependent manner. We propose three inter-related specific aims for this problem.

1.2.1. Investigate mechanisms of indole mediated anti-inflammatory signaling in macrophages and epithelial cells

In this Aim, we utilize epithelial and macrophage cell lines and primary cells to characterize the effects of indole on pro-inflammatory cytokine production. We further investigate the mechanisms (receptors and signaling pathway) underlying modulation of inflammation using a range of genetically modified knockout cells and chemical inhibitors. Lastly, we also quantify changes in microRNA (miRNA) gene expression as a novel mechanism for indole-mediated modulation of inflammation in the above-mentioned cell types.

1.2.2. Investigate mechanisms of anti-inflammatory potentiation of a synthesized indole derivative

Based on prior work showing that indole signaling is AhR-dependent in mouse and human epithelial cells but independent of the AhR in murine macrophages, we seek to delineate the receptor and downstream signaling elements involved in mediating indole signaling. To further investigate mechanistic signaling of indole, we develop a synthesis scheme for generating a cell-impermeant indole derivative and use it to test the contribution of cell-surface mediated signaling in the observed attenuation of inflammation. We characterize the anti-inflammatory effects of this novel compound in

macrophages and utilize transcriptome profiling to identify signaling pathways specific to this molecule.

1.2.3. Investigate mechanisms of indole and 5-hydroxyindoles effects on T cell differentiation

Prior work has shown that two MDTMs, indole and 5-hydroxyindole, augment or inhibit the differentiation of naïve T cells into Tregs and Th17 cells, respectively. However, the underlying mechanisms and pathways are not known. In this Aim, we use transcriptome profiling to characterize the mRNA signature of T cells that are differentiated into Tregs or Th17 in the presence of indole and 5-hydroxyindole.

1.3. Innovation and Significance

While the contribution of the microbiota to human health and disease is well documented, a mechanistic understanding of their effect is lacking[4,7]. In this work, we demonstrate the anti-inflammatory effects of various MDTMs in cell contexts that have not previously been reported. We also identify a possible novel role for miRNA-mediated effects in MDTM modulation of inflammation in macrophages. The synthesis of an indole derivative for specifically investigating cell surface interactions of MDTMs is also novel and not been previously described. This research reagent will enable future studies to partition intracellular vs cell-surface mediated effects of MDTMs. Similarly, this study goes beyond phenotypic characterization of responses to identifying signaling pathways involved in two immune cell types: macrophages and T cells. In macrophages,

we identify an axis between the transcription factor, NRF2, and indole signaling. In T cells, we find that indole augments regulatory T cell differentiation and identify the transcription factor, IRF3, as a novel mediator for indole signaling. These results lay the foundation for further investigation into IRF3 and type I interferon signaling in Treg differentiation and function.

2. LITERATURE REVIEW

2.1. Human Microbiota

The human body plays host to mutualistic microbes with some major sites of colonization being the gut, the oral cavity, and the skin[62]. Mutualistic skin microbial communities can have large inter-individual variability, however, longitudinal studies find that populations within individuals remain relatively stable over time[63]. Various ecological niches of the skin harbor adapted microbes, with oily skin regions like the face being dominated by lipophilic bacteria, i.e., members of the genus *Propionibacterium*, and moist skin regions like the crook of the elbow being dominated by bacteria that thrive in high humidity environments, i.e., the genus *Staphylococcus*. Symbiotic microbes at the epidermis can function to train the cutaneous immune system and inhibit colonization by pathogens, with the common colonizer *Staphylococcus epidermidis* inhibiting colonization by the opportunistic pathogen *Staphylococcus aureus*[64,65]. Culture supernatants from *S. epidermidis* inhibited production of pro-inflammatory cytokines in wounded mouse skin, with the component lipoteichoic acid alone demonstrating potent inhibitory activity *in vitro*[66].

More than 700 species have been identified in the human oral microbiome and dental plaques are estimated to harbor roughly an order of magnitude more bacteria than the skin[1,67]. Two critical sites for the development of the common diseases, periodontitis and dental caries, are the subgingival crevice and the tooth surface[68,69]. For a given individual, the subgingival community consists of ~60 high abundance species, while the

tooth surface is dominated by ~14 species[67]. Facultative anaerobes of the genera *Streptococcus* and *Gemella* are commonly found at both sites, while the aerobic *Rothia dentocariosa* preferentially colonizes the tooth surface. In the development of dental caries, dysbiosis at the tooth surface can be triggered by various factors, including increased consumption of fermentable sugars[68]. The increased exposure to nutrients can drive commensals to become opportunistic pathogens, leading to expansion of biofilm communities on the tooth and increase in localized acid production. The high pH environment leads to demineralization of tooth enamel and selection for acidophilic colonizers, leading to dysbiosis and the development of dental caries. Hypotheses for the development of periodontitis mirror a similar pathology but inducing dysbiosis in communities within the subgingival crevice. While individual species have been associated with dental caries and periodontitis, i.e. *Streptococcus mutans* and *Porphyromonas gingivalis*, respectively, the oral microbiome in disease are commonly characterized in the absence of these keystone species, suggesting the conditions are a result of ecological niches developed in dysbiosis rather than being caused by individual pathogens[70]. Furthermore, administration of *P. gingivalis* in antibiotic treated mice alone was unable to induce periodontitis, while pre-colonization with the common commensal *Streptococcus gordonii* followed by *P. gingivalis* lead to alveolar bone loss[71].

The colonic microbiota is estimated to harbor two orders of magnitude more bacteria than the oral microbiome[1]. In addition to this, the ileum, jejunum, and duodenum are also major sites of colonization in the GI tract. Inter-individual variation in compositions is large, but nonetheless, the phyla Firmicutes and Bacteroidetes have

been found to be the most abundant in human fecal samples, representing roughly 60% of the microbiota[72]. Within the phylum Firmicutes, the genera *Faecalibacterium* and *Lachnospiraceae* are most abundant, while *Bacteroides* are the predominant genus within the Bacteroidetes phylum. These compositions as well as total populations vary along the intestinal tract, with a trend of increasing abundance traversing from the duodenum to the distal colon[1]. While the colon and ileum contain large populations of the phylum Bacteroidetes, the jejunum is composed largely of the phylum Firmicutes, in particular *Streptococci*, and the phylum Proteobacteria[73,74]. Though the colon and ileum bear similarities, the colonic microbiota contains a larger proportion of the *Clostridium* cluster XIVa. These differences exist in part due to oxygen gradients along the GI tract, with levels being higher towards the duodenum and diminishing towards the colon[75]. This may explain the selection for the dominant facultative anaerobes in the jejunum, and the dearth of extreme obligate anaerobes that are otherwise abundant in the colon, e.g., *Faecalibacterium prausnitzii.* Another selective factor along the GI tract is the relative abundance of various nutrient resources. The small intestine has high exposure to carbohydrates digestible by both the host and microbiota. In contrast, the colon receives the resistant starches that are not digested by the host or the small intestinal microbes, and hence are substrates for SCFA synthesis by the colonic bacteria.

2.1.1. Intestinal microbiota dysbiosis

Dysbiosis or alterations in the composition of the intestinal microbial community has been associated with several diseases, both in the GI tract and elsewhere in the body.

Diseases that are reported to have a microbial component include obesity, T2D, atherosclerosis, cardiovascular disease, autism spectrum disorder, Parkinson's disease, depression, Crohn's disease, colitis, rheumatoid arthritis, colorectal cancer, and NAFLD[8-19]. While a majority of the studies have reported associations between changes in the composition and disease, whether these associations are causative in nature is not fully understood[76]. Because of a limited ability to manipulate and measure changes in the native gut microbiota, many experiments to-date have been correlative in nature. However, experiments that recapitulate disease phenotypes by transfer of the disease-associated microbiota to germ-free or gnotobiotic mice have been used to infer causative roles of the microbiota. For example, transfer of dysbiotic microbiota from diet-induced obese mice or penicillin-treated mice into germ-free mice resulted greater mass and fat gain relative to the transfer of unperturbed microbiota[17,27]. Similarly, transplantation of fecal microbiota from humans with major depressive disorder resulted in depression-like behavior in germ-free mice compared to mice colonized with the fecal microbiota of healthy patients[28]. Mendelian randomization has also been used to infer a causal relationship between (i) the SCFA, propionate and risk of T2D, (ii) abundance of the family Oxalobacteraceae and protection from rheumatoid arthritis, and (iii) abundance of the genus *Bifidobacterium* and protection from ulcerative colitis[29,30].

2.1.1.1. Microbiota and obesity

The global prevalence of obesity has increased in the past 50 years and projections of obesity prevalence in the United States estimate 48.9% of adults will be

obese by 2030[77,78]. A defining feature of obesity is excess adiposity and is a risk factor for metabolic and cardiovascular diseases. Alterations in gut microbial composition and decreased diversity of the microbiota have been associated with obesity[17,27,79-83]. The fecal metagenome of obese individuals is characterized by lower diversity, lower richness of microbial genes, and increase in the capacity for energetic metabolism of dietary compounds relative to non-obese individuals[17,82]. At the phylum level, the abundance of Firmicutes relative to Bacteroidetes is increased in obese mice and human patients, though contradictory results have been reported[17,84]. The presence and abundance of *Akkermansia muciniphila* that belongs to the phylum Verrucomicrobia has also been correlated with decreased body weight and obesity in humans and mice[85]. Despite some conflicting evidence with the microbiota associations, obesity-associated microbiota have demonstrated transmissibility, with germ-free mice colonized by the fecal microbiota of obese mice or humans having greater increases in adiposity[17,81]. Co-housing of obese and lean littermates leads to increased colonization by Bacteroidetes in obese mice that correlated with a decrease in obesity phenotypes[81]. Interventions in mice via oral gavage with *Bacteroides thetaiotamicron* or *A. muciniphila* attenuated diet-induced weight gain and improved metabolism in obese mice[80,86]. Interestingly, a clinical trial in overweight or obese volunteers, supplementation with heat-killed *A. muciniphila* decreased weight and fat mass, while supplementation with live cultures did not induce significant changes[87]. Dietary intervention in overweight or obese individuals with a energetically-matched Mediterranean diet led to concurrent changes in microbial composition, with increases in *Bacteroides uniformis* and *Bacteroides vulgaris*, and

improvement in blood cholesterol levels[79]. These results suggest the gut microbiota may have causative contributions to obesity, and that dietary and probiotic interventions may have therapeutic potential.

2.1.1.2. Microbiota and inflammatory bowel disease

Inflammatory Bowel Diseases (IBD) are intestinal disorders that are often typified as Crohn's disease (CD) or ulcerative colitis (UC) and involve chronic gut inflammation[88]. In Europe and North America, IBD has a prevalence of roughly 800 cases and 600 cases per 100,000, respectively, and incidence in some more recently industrialized countries has been on the rise for decades[89]. While the precise etiology of these diseases remain to be elucidated, symptoms are known to arise from multi-directional signaling between the gut microbiota, the host, and environmental factors[90]. Analysis of colonic material from IBD patients reveal decreased abundance of Firmicutes and Bacteroidetes and increased abundance of Fusobacteria and Proteobacteria[11,24,91]. While causation can be difficult to prove, particularly in complex systems with bidirectional signaling, a Mendelian randomization study design was used to elucidate the causal association between low abundance of the phylum Actinobacteria and UC[30]. Significant alterations in many classes of microbially-derived or microbially-modified compounds are also observed in diseased states, particularly a decrease in SCFAs, and increase in acylcarnitines and primary bile acids[91]. Host genetics can also directly affect gut microbial composition and metabolite composition. The knockout of a gene with known susceptibility loci for IBD, caspase recruitment domain family member

9 (CARD9), significantly altered gut microbial composition and the presence of MDTMs in mice[92]. While IBD is currently an incurable condition, microbiota-targeted interventions have shown promise for amelioration of disease. Administration of *F. prausnitzii* or its culture supernatants attenuated experimental colitis and repaired dysbiosis in mice[93]. Fecal microbiota transfer (FMT) from healthy donors into patients with active UC induced disease remission and increase gut microbial diversity[94]. Dietary interventions that altered the microbiome of children with mild to moderate CD have also been able to attenuate disease[95].

2.1.2. Microbially-derived metabolites

A healthy gut microbiota provides the host with several functional benefits, including maintaining intestinal homeostasis via protective activation of toll-like receptors (TLRs), detoxification of xenobiotic compounds, and the metabolism and synthesis of dietary metabolites belonging to a wide range of chemical classes[96-98]. In particular, the metabolism of polysaccharides that are otherwise indigestible by the host organism is critical in energy homeostasis, and the resulting metabolic activity results in the synthesis or secretion of multiple compounds, including essential amino acids such as tryptophan, vitamins, secondary bile acids, choline derivatives, SCFAs, and MDTMs[15,33,38,98-101]. It is now widely accepted that metabolites produced by the microbiota are involved in mediating a majority of the beneficial effects associated with the microbiota.

2.1.2.1. Short-chain fatty acids

The best studied class of microbial metabolites is SCFAs, the obligate products of anaerobic fermentation of host-indigestible polysaccharides[35]. The colon epithelial cells of humans and other mammals supply roughly 70% of their energy intake via the oxidation of these compounds, which is roughly 10% of the total caloric intake[98]. Concentrations of total SCFAs in the proximal colon in healthy adults are in roughly the 100 mM range, with a wide margin of variability based on diet and environmental factors[35,102]. The primary SCFAs produced by gut microbial fermentation are acetate, propionate, and butyrate, with acetate typically being the most abundant[35]. A decrease in the abundance of butyrate-producing bacteria in the GI tract has been correlated with T2D, while a diet high in indigestible polysaccharides was shown to increase the abundance of SCFA producing bacteria in the colon and attenuate T2D[16,103]. Similarly, a decrease in the levels of butyrate-producing bacteria such as *F. prausnitzii* and *Roseburia intestinalis* have been correlated with IBD[93,104]. Combined treatment with mixtures of the major SCFAs have been shown to increase the abundance and homeostatic function of colonic regulatory T cells (Tregs), a critical immune cell in regulating gut inflammation[56]. Individual treatment with butyrate has been shown to promote peripheral Treg generation, augmenting differentiation of naïve $CD4^+$ cells into Tregs and attenuating T-cell dependent experimental colitis in mice[105,106]. Similarly, propionate also augmented peripheral Treg differentiation, reduced splenic Th17 populations in murine models of hypertensive cardiovascular damage, and simultaneously increased Treg populations while decreasing Th17 populations in

multiple sclerosis patients[107,108]. While acetate was not found to promote peripheral Treg generation in standard mice, acetate treatment was found to increase Treg populations and suppressive capability after induction of allergic airway disease[109]. In contrast to microbially produced SCFAs, long chain fatty acids (LCFAs) are abundant directly from dietary sources. In a murine model of multiple sclerosis, LCFA supplementation decreased intestinal SCFA levels that correlated with an increase in intestinal Th1 and Th17 populations[110]. Clinical interventions for IBD based on SCFA modulation have demonstrated mixed results, however, some of the positive outcomes from SCFA enemas and the promotion of SCFA production via prebiotics or probiotics warrant further investigation into therapeutic potential of SCFA modulation in Crohn's disease and ulcerative colitis[104].

2.1.2.2. Secondary bile acids

Cholesterol is metabolized into primary bile acids by the liver[111]. Secreted primary bile acids can be transported to the GI tract where enzymes of microbial origin catalyze further modifications to yield secondary bile acids. Gut dysbiosis and subsequent perturbation of secondary bile acid levels have been correlated with pathologies including liver diseases, cancers, and pathogenic infection[112-117]. In antibiotic treated mice, adoptive transfer of the bile acid metabolizing commensal *Clostridium scindens* inhibited subsequent *Clostridium difficile* infection. This correlated with a concomitant increase in the relative abundance of genes involved in the biosynthesis of secondary bile acids and was ablated by co-administration with the bile

acid sequestrant cholestyramine, suggesting antimicrobial properties of the secondary bile acids themselves[116]. Conversely, increased levels of circulating secondary bile acids have been associated with NAFLD and non-alcoholic steatohepatitis[112,113]. Furthermore, secondary bile acids inhibited *Cxcl16* chemokine expression and subsequent recruitment of natural killer T cells with anti-tumor properties in various murine models of liver cancer[114]. In addition, *Cxcl16* expression was inversely correlated with levels of the secondary bile acid, glycolithocholate, in human liver samples from liver cancer patients. Metagenomic analyses also identify enriched expression of bile acid enzyme expression in fecal samples from colorectal cancer patients, further implicating a role for secondary bile acids in cancer[115].

2.1.2.3. Tryptophan metabolites

Tryptophan is an essential amino acid that cannot be produced by mammalian hosts and needs to be supplied through the diet. Dietary tryptophan is metabolized by both the mammalian host and the gut microbiota. More than 95% of tryptophan is relegated to the host-mediated kynurenine pathway that ends in the production of NAD^+ (**Figure 2.1**) [51]. The other major pathway in mammalian tryptophan metabolism is the serotonin pathway. The vast majority of serotonin in the human body is produced by enterochromaffin cells in the in the lumen of the GI tract. Serotonin is a neurotransmitter that is involved in several biological processes including cognition, food intake, and emotional processing[118]. Tryptophan that does not get absorbed in the small intestine is metabolized by the microbiota in the colon to form a broad range of MDTMs that

contain an indole moiety (**Figure 2.1**). Indole is produced as a result of tryptophanase activity that is encoded by the TnaA gene in many bacteria. More than 80 bacterial species including *Bacteriodes thetaiotaomicron* have been documented to produce high levels of indole[119]. Analysis of the human metagenome identified more than 300 bacterial genes with putative tryptophanase activity. These genes were distributed largely in uncharacterized commensals, and bacteria belonging to the phyla Bacteroidetes and Firmicutes[120]. The reaction catalyzed by tryptophanase results in the generation of indole, ammonia, and pyruvate as an energy source[121]. If tryptophan availability is limiting, competition between the kynurenine, serotonin, and microbial indole pathways may be one mechanism facilitating cross-talk along the gut-brain axis[122]. Low tryptophan levels and elevated serum kynurenine to tryptophan ratio are associated with Crohn's disease[123,124].

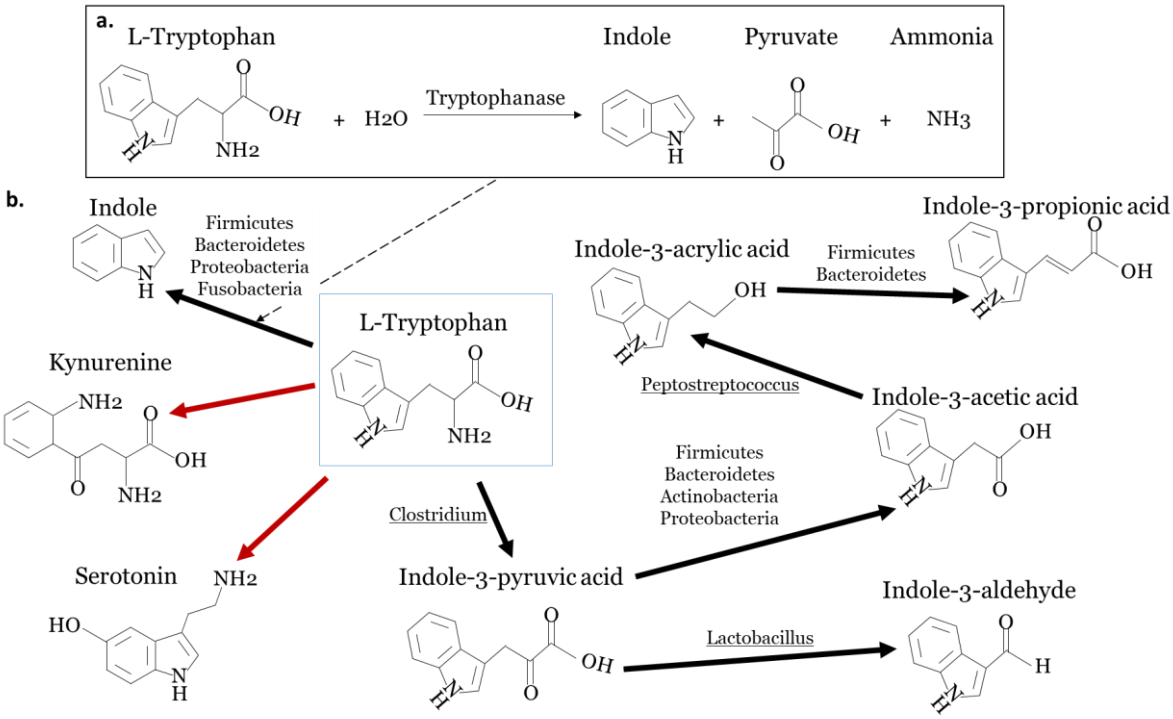


Figure 2.1. Microbially-derived tryptophan metabolites. (a) Bacterial tryptophanases catalyze the conversion of L-tryptophan to indole, pyruvate, and ammonia. **(b)** Known metabolites of L-tryptophan in the human body. Compounds with black arrows are microbially-derived while compounds with red arrows are host-derived. Bacterial phyla and genera known to produce a metabolite are listed, with genera underlined.

Studies have found median fecal indole concentrations in human fecal samples to be approximately in the low millimolar range[57-60], with one study reporting values ranging from 0.3 to 6.64 mM in healthy adults[58]. The indole that is generated from tryptophan can be further modified into other metabolites, such as hydroxyindoles, indoxyl sulfate, and MDTMs, such as indole-3-propionic acid and indole-3-aldehyde (IAld), can be derived from tryptophan through alternative pathways[47,125]. Formation of some of indole metabolites are completely host dependent: conversion of indole to

indoxyl is mediated by mammalian cytochrome P450 2E1, followed by sulfotransferase 1A1 mediated sulfation to form indoxyl sulfate[126,127]. Conversely, some of the biotransformation reactions involving indole are present in both the microbiota and the host: mammalian CYP11A1 can directly covert indole to 5-HI while gut bacteria can convert 5-hydroxytryptophan to 5-HI[128,129].

Production of MDTMs in the GI tract is likely to involve co-metabolism between the microbiota and the host. For example, while indoxyl sulfate biosynthesis from indole is purely host dependent, formation of indole itself is microbiota dependent, resulting in abrogation of circulating indoxyl sulfate levels in germ free mice[47]. It should be noted that multiple routes exist to produce MDTMs. For example, members of the genus *Lactobacillus* can directly produce IAld without the production of indole[42,45,125,130,131]. Similarly, tryptamine can be produced directly from tryptophan through the tryptophan decarboxylase enzyme and multiple enzymatic routes to produce indole-3-acetate (both from tryptophan and indole) have been recently reported[132,133].

Several *in vitro* and *in vivo* studies have demonstrated a role for indole in modulating inflammation in multiple models, ranging from suppressing diet-induced non-alcoholic fatty liver disease and liver inflammation, to protecting against dextran sodium sulfate induced colitis and promoting intestinal epithelial function, to reducing central nervous system inflammation in a murine model of multiple sclerosis[39,40,42,48,49]. Administration of IAld in mice also attenuated experimental colitis, inhibited fungal growth in candidiasis, alleviated central nervous system inflammation in experimental

autoimmune encephalomyelitis, and inhibit skin inflammation in a dermatitis model[42,45,125,130,131].

2.1.2.4. Trimethylamine metabolites

Choline, phosphatidylcholine, carnitine, and betaine are among the dietary compounds that the gut microbiota can utilize as a carbon source, releasing trimethylamine (TMA) as a by-product of metabolism[14,15]. TMA-monooxygenases, primarily expressed in the mammalian host liver, oxidize TMA to trimethylamine-N-oxide (TMAO). While a TMA-monooxygenase has been characterized in the methanotrophic species *Methylocella silvestris*, such enzymatic activity has not been identified in abundant human symbionts, suggesting microbial dependent effects on TMAO levels may be mediated primarily through TMA synthesis[134]. Plasma levels of TMAO have been associated with risk of cardiovascular disease (CVD), atherosclerosis, and thrombotic events[14,15,52-54]. Dietary supplementation of TMAO in mice promoted atherosclerosis, reduced reverse cholesterol transport, and stimulated platelet hyper-reactivity[14,15,54]. However, at least one study did not find an association between plasma TMAO levels in mice and atherosclerosis, but did observe a correlation with plaque instability[135]. In support of a causative role of the microbially dependent TMAO levels and CVD, transfer of cecal microbiota from pro-atherosclerotic mice into choline-fed germ-free mice induced faster thrombus formation than the transfer of a low-TMAO producing microbiota[54]. Outside of CVD, an overabundance of choline-TMA lyase genes was observed in the fecal microbial metagenome of colorectal cancer patients[136].

2.2. Macrophages

Macrophages are immune effector cells that are the most abundant immune cell type in the intestinal lamina propria[137]. Macrophages can arise from terminal differentiation of circulating monocytes and infiltrate tissue. In addition, resident macrophages are also seen in many tissues in the body, for example, Kupffer cells in the liver, microglia in the brain, and adipose tissue macrophages. However, tissue-resident and circulating macrophage populations can differ in their origin. While monocytes in peripheral blood originate from bone-marrow derived progenitor cells, some tissue-resident macrophages are independently maintained populations originating from yolk-sac derived cells in early life[138]. These localized macrophages develop highly specialized functions. For example, intestinal macrophages contributing to wound healing and the proliferation of the epithelium as well as maintaining tolerance to commensals and symbionts[139].

In general, macrophages function by secreting signaling cytokines, phagocytosing pathogens and damaged host cells, and presentation of appropriate antigens for activation of other immune cells. Circulating monocytes and macrophages migrate towards exogenous molecular patterns, such as LPS signaling the presence of potentially pathogenic bacteria, and endogenous molecular patterns, such as heme released from damaged erythrocytes in need of phagocytic recycling[140]. Chemokines secreted by other immune cells can also induce monocyte/macrophage migration and adhesion. Monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) are two chemokines that are produced by different cell types including epithelial cells and

macrophages themselves and are typically upregulated in pro-inflammatory environments[141,142]. Like the aforementioned molecular patterns, MCP-1 is a strong inducer of macrophage/monocyte chemotaxis to sites of inflammation while IL-8 is involved in macrophage adhesion.

Macrophages may also be further polarized after differentiation and are often characterized as either classically (M1) or alternatively (M2) activated[143]. This polarization is often driven by T-cell derived cytokines, with T-helper 1 (Th1) cells producing interferon-γ (IFN- γ) and T-helper 2 (Th2) cells producing interleukin 4 (IL-4). The combination of LPS and IFN- γ are strong drivers for pro-inflammatory M1 polarization with increased capacity to secrete pro-inflammatory cytokines and pathogen clearance, while IL-4 drives M2 polarization and a wound-healing associated phenotype with increase capacity to secrete the anti-inflammatory cytokine interleukin-10 (IL-10)[143].

Aberrant M1 macrophage activity can induce tissue damage at sites of inflammation and be pathogenic in immune-driven diseases. Macrophage mediated chronic low-grade inflammation is a hallmark of diet-induced obesity[144,145]. Adipose tissues of obese individuals express increased levels of MCP-1, inducing macrophage infiltration and proliferation within adipose tissues[146]. This increase in MCP-1 secretion can be induced by the gut microbiota via a mechanism dependent on toll-like receptor 4 (TLR4), the primary receptor for LPS recognition[147]. Obese individuals are estimated to have adipose tissues with a population comprised of roughly 40% macrophages, while lean individuals may have less than 10%[148]. Obesity can induce polarization of

infiltration macrophages towards the M1 phenotype, resulting in increased secretion of tumor necrosis factor (TNF) and other pro-inflammatory cytokines capable of promoting insulin resistance, a defining characteristic of T2D and metabolic syndrome[145].

Dysregulation of intestinal macrophages have been implicated as a causative factor in IBD. Intestinal macrophages are a heterogeneous population and can originate from circulating monocytes as well as self-maintained populations[149,150]. Gut microbial signals are critical for maintaining homeostatic levels of monocyte-derived intestinal macrophages, as germ-free mice are significantly depleted in these populations[149]. These localized macrophages play a critical role in maintaining tolerance to gut symbionts and dietary antigens, in part by IL-10 secretion, driving regulatory T cell (Treg) expansion, and limiting epithelial absorption of exogenous toxins[151-153]. Genetic analysis of IBD patients reveal enrichment of loci associated with monocyte differentiation or activation and macrophage-LPS response[154]. Similarly, susceptibility genes for IBD are differentially expressed in macrophages after phagocytic sampling of apoptotic epithelial cells, suggesting functional deficiencies in efferocytosis in the gut may contribute to etiology[155]. Function and differentiation of macrophages can be directly regulated by microbially-derived metabolites, with butyrate driving macrophage differentiation from circulating monocytes, inhibiting LPS-induced nitric oxide, IL-6, and IL-12 secretion, while increasing pathogen resistance[156,157].

In addition to butyrate, the MDTM indole-3-acetate inhibits LPS-stimulated expression and secretion of pro-inflammatory TNF and MCP-1 [44]. Furthermore, ablation of the AhR in CD11c expressing cells (primarily expressed in dendritic cells and

macrophages) resulted in epithelial barrier dysfunction and increased susceptibility to experimental colitis[158].

Taken together, there is significant evidence that macrophages are a critical immune effector cell type involved in the etiology of multiple inflammation-mediated diseases, and gut microbial dysbiosis can contribute to aberrant macrophage function and differentiation, in part through altered microbial metabolite signaling.

2.3. T Cells

T cells comprise a diverse population of immune effector and helper cells critical for maintenance of adaptive immune responses and immune memory. They originate from bone marrow progenitor cells that migrate to the thymus where they begin to express the T-cell receptor (TCR), CD4, and CD8[159]. The ability of T-cells to respond to a massive repertoire of exogenous antigens is dependent on the diversity of TCR sequences. Segments of the TCRα and TCRβ genes of immature T-cells undergo rearrangement facilitated by recombination activating gene (RAG) 1 and 2 while in the thymus, with the theoretical potential to generate on the order of 10^{15} unique TCRs[160]. These T-cells undergo positive selection for their ability to bind major histocompatibility complex (MHC) class I or class II molecules presented by thymic epithelial cells, that then drive differentiation into $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ subtypes[161,162]. MHC class I molecules are expressed at the cell surface by all nucleated cells and present intracellular protein fragments, whereas MHC class II molecules are primarily present at the surface of antigen presenting cells, displaying exogenous antigens. Affinity for MHC class I

drives $CD4^{-}CD8^{+}$ differentiation, while affinity for MHC class II drives $CD4^{+}CD8^{-}$ differentiation[163]. These single positive T-cells then undergo negative selection for TCR affinity towards self-antigens presented by thymic dendritic cells (DCs), with high affinity binding initiating apoptosis. This combination of positive selection for MHC binding and negative selection for self-antigen recognition selects for helper ($CD4^{+}$) and cytotoxic ($CD8^{+}$) T cell populations that are immunocompetent while remaining self-tolerant[162]. After affinity maturation, young adults are estimated to have a lower bound of 100 million unique TCR binding sequences, allowing for broad yet targeted recognition of foreign antigens[164]. Affinity-matured T cells can migrate from the thymus to the spleen, lymph nodes, mucosal sites, or persist in circulation[159]. In the periphery, cognate antigen recognition drives clonal expansion, further differentiation into specialized subsets, and cytokine secretion. For example, $CD8^{+}$ cytotoxic T cells secrete cytotoxic compounds such as perforin and granzymes to facilitate pathogen clearance, while $CD4^{+}$ helper cells secrete cytokines to signal immune status[165,166]. Post-infection, most of the expanded T-cells undergo apoptosis, while a small subset persists as memory T-cells that function to maintain long-term immunity to subsequent pathogen exposure.

Within the helper T cell lineage, there exists four major subpopulations: T-helper 1 (Th1), T-helper 2 (Th2), T-helper 17 (Th17), and T-regulatory (Treg) cells (**Figure 2.2**). When naïve $CD4^{+}$ T cells (Th0) are activated by DCs presenting its cognate antigen, the fate of its differentiation into a given subtype is largely determined by the presence and concentration of cytokines within its vicinity[165].

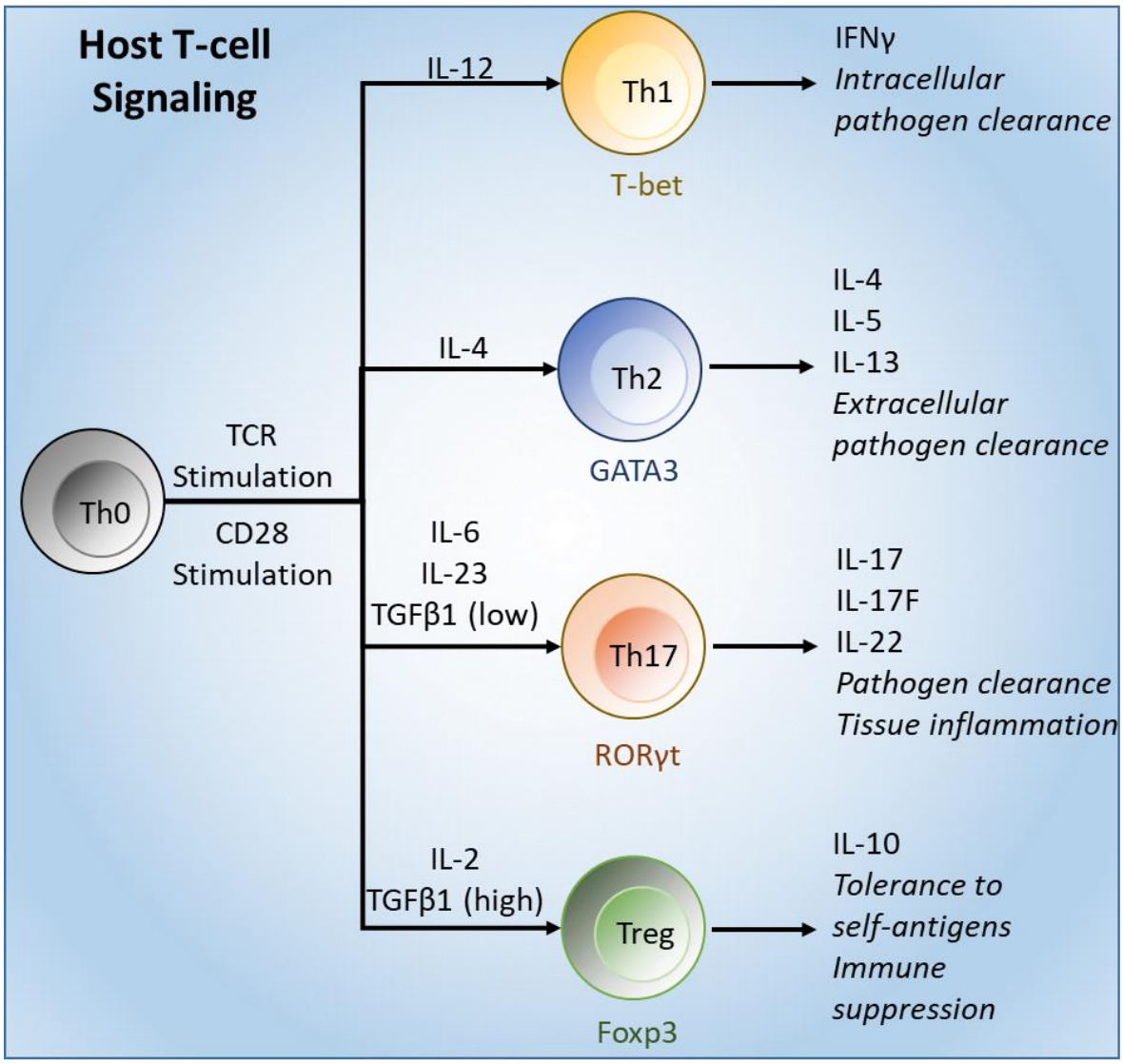


Figure 2.2. Ex vivo helper T cell differentiation. Activation of naïve $CD4^+$ T cells by antigen presenting cells can be simulated *ex vivo* using antibodies for TCR co-receptors, CD3 and CD28. Addition of recombinant cytokine milieus drive differentiation towards specific subtypes, with four major lineages depicted. Master transcription factors for each type are depicted along with functions and classical cytokines produced.

Th1 differentiation is driven by the presence of interleukin 12 (IL-12) that can be produced by DCs and macrophages, and the presence of IFN- γ from natural killer cells and Th1 cells themselves, resulting in positive feedback[167]. Differentiation is

mechanistically driven by the activation of the T-box transcription factor (T-bet), which also actively suppresses the expression of the master transcription factors for other subtype lineages as well as expression of cytokines needed for differentiation into those lineages. Th1 cells promote inflammatory responses against intracellular pathogens through the secretion of cytokines. In addition to further promoting Th1 differentiation, IFN- γ also contributes to classical macrophage activation and enhanced phagocytic activity. Another major Th1 cytokine, interleukin 2 (IL-2), promotes proliferation of $CD8^+$ cytotoxic T cells.

Th2 differentiation is driven by IL-2 and interleukin 4 (IL-4) that can be produced by naïve T cells, as well as Th2 cells themselves, again mimicking the feedback mechanisms present in Th1 differentiation[168,169]. Under the control of master transcription factor GATA3, Th2 can function to promote alternative macrophage activation, clearance of extracellular pathogens and parasites, and secret anti-inflammatory IL-10[170].

Th17 differentiation is driven by interleukin 6 (IL-6), a major cytokine produced by inflammatory macrophages and low levels of TGF-β, that can be produced by macrophages and other immune cells[171]. The presence of interleukin-23 promotes maintenance and expansion of differentiated Th17 cells and are produced primarily by DCs and macrophages. Primarily under the control of the transcription factor RORγt, Th17 cells secrete its defining cytokine, interleukin 17A (IL-17A), as well as interleukin 17F (IL-17F), and interleukin 22 (IL-22)[172]. Similar to Th1 cells, Th17 cells are involved in the response to pathogens, however some bacteria, including *Klebsiella pneumoniae*,

Mycobacterium tuberculosis, and *Pneumocystis carinii,* can specifically stimulate IL-17 production or require IL-17 for optimal defense[173]. As IL-17 and IL-22 receptors are expressed in a wide range of cell types including epithelial and endothelial cells, the development of Th17-mediated immunity may have evolved from a need for a more potent response than Th1-mediated immunity can supply[171]. In addition to being induced by bacterial pathogens, memory T cells against fungal pathogens are predominantly of the Th17 subtype, suggesting a critical role in immunity against fungi[174].

Induced regulatory T cell (iTreg) differentiation is driven by the combination of IL-2 and high concentrations of TGF-β. Unlike the other subtypes described here, the primary function of Tregs is in immune tolerance and the resolution of inflammation[175]. Notably, IL-2 is secreted by various T cells, and TGF-β is also a required cytokine for Th17 differentiation, highlighting the complex crosstalk between these cytokines that facilitate adequate immune response to pathogenic insults, while preventing aberrant inflammation. Tregs are under the control of the forkhead box P3 (Foxp3) transcription factor that drives IL-10 secretion and programs its function in limiting proliferation of pro-inflammatory immune cell types[176].

At homeostasis, the gut microbiota can affect health by influencing the activity and differentiation of T cells. Germ-free mice mono-colonized by the commensal bacterium, *Candidatus savagella,* resulted in the presence of Th17 cells in the lamina propria, providing colonization resistance against challenge with pathogenic *Citrobacter rodentium*[177]. Mixtures of SCFAs or butyrate alone have demonstrated the ability to increase differentiation, abundance, and proportions of Tregs in the murine colon[56,105]. In

addition, SCFA feeding alleviated severity of experimental colitis, suggesting this class of microbially dependent metabolites have Treg-promoting potential. Despite this, clear mechanistic pathways mediating SCFA-induced changes in T cells and the effects of other major classes of microbially-derived metabolites have yet to be well defined. In this work, we investigate the effects of MDTM on helper T cell differentiation, with a focus on Th17 and Treg populations, and further utilize transcriptome profiling to identify gene expression signatures induced by MDTM treatments.

2.4. The Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor (AhR) is sequestered in the cytosol in an inactive state by heat shock protein 90 (HSP90) and X-associated protein 2 (**Figure 2.3**)[178]. Binding of an agonist, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or benzo[*a*]pyrene (B[*a*]P), leads to its nuclear translocation and binding to xenobiotic response elements (XREs), also known as dioxin response elements, in the promoter region of AhR responsive genes such as Cytochrome P450 Family 1 subfamily A member 1 (*Cyp1a1*). TCDD is used as the prototypical AhR agonist, and along with the endogenous AhR ligand, 6-formylindolo[3,2-b]carbazole (FICZ), have the highest AhR binding affinities quantified. In a radiolabeled competitive binding assay, TCDD and FICZ were both found to have an IC_{50} of 1 nM, the lowest in a panel of known AhR ligands[179]. Included in this panel are multiple ligands with significantly differing chemical characteristics, including pentacyclic benzo[*a*]pyrene, the flavonoid beta-naphthoflavone, and the bis-indole indirubin. All ligands tested demonstrate potent AhR-

agonism and illustrate the promiscuous nature of the AhR in recognizing a diverse repertoire of ligands.

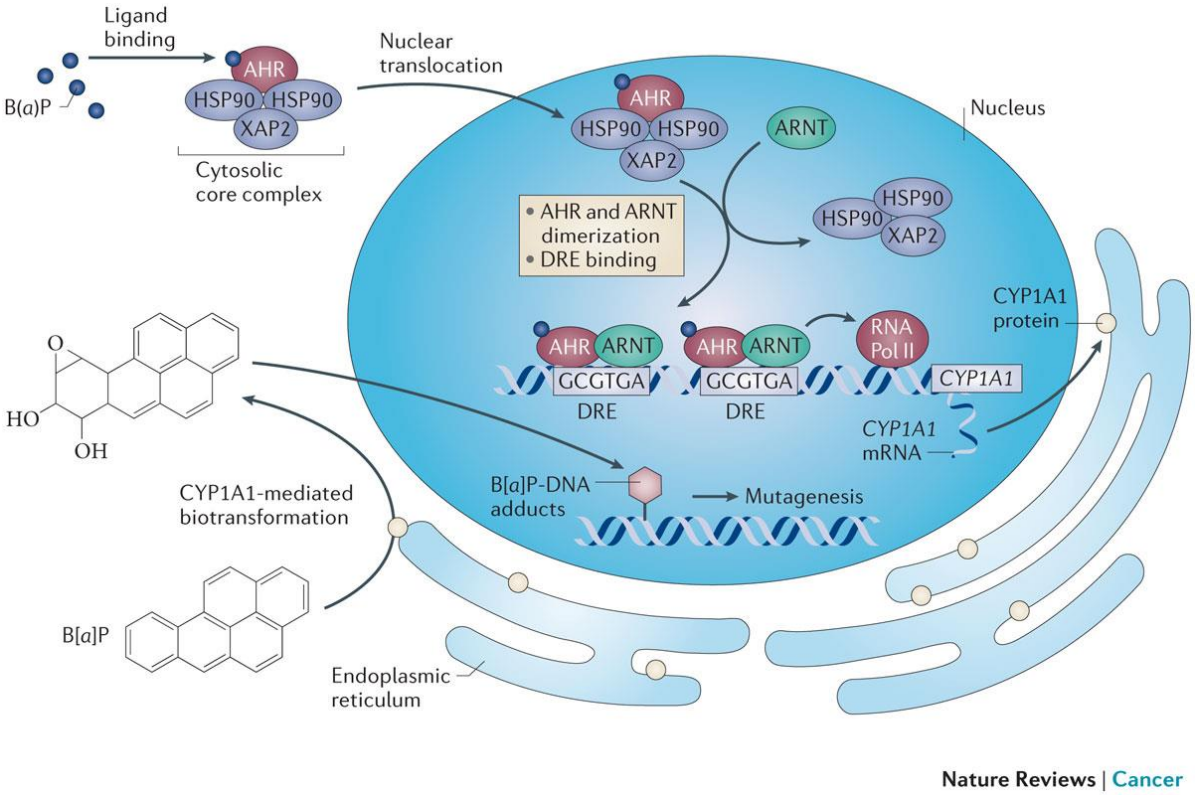


Figure 2.3. AhR transcription factor signaling. Inactive AhR is sequestered in the cytosol in a complex with heat shock protein 90 (HSP90) and X-associated protein 2 (XAP2). Ligand binding, i.e. to benzo[*a*]pyrene (B[*a*]P), facilitates interaction with the AhR nuclear translocator (ARNT), release from the cytosolic complex, and translocation to the nucleus. Here, the AhR-ARNT complex binds to dioxin-responsive elements (DREs), upregulating transcription of targets genes, such as phase I metabolism genes, i.e., cytochrome P450 1 subfamily A member 1 (*Cyp1a1*), capable of metabolizing some AhR ligands.

Similarly, while *Cyp1a1* is used as the prototypical AhR-responsive gene, alternative targets include other members of the cytochrome P450 family (e.g., *Cyp1b1* and *Cyp1a2*), and phase II detoxifying enzymes (NAD(P)H: quinone oxidoreductase (*Nqo1*), glutathione S-transferase alpha 1 (*Gsta1*) and other glutathione S-

transferases)[180,181]. Induction of these genes involved in xenobiotic metabolism by multiple ligands facilitates the canonically recognized function of the AhR which is regulating the response to environmental contaminants[182]. Subsequently, a role for the AhR in metabolizing natural compounds, endogenous tryptophan-derived products, and in modulating the immune system has been proposed[183-185].

Our previous work demonstrated that the MDTMs indole, indole-3-acetate, and tryptamine are ligands for the AhR[186,187]. Indole behaves as a weak AhR agonist in Caco-2 colon epithelial cells, with 1 mM concentrations inducing *Cyp1a1* to roughly half the level induced by 10 nM TCDD. However, it also behaves an antagonist of TCDD-mediated *Cyp1a1* induction, with co-incubation with 500 μM indole and TCDD reducing *Cyp1a1* expression by 2-fold relative to TCDD alone. On the other hand, I3A and tryptamine only showed AhR agonist activity, but lacked antagonism of TCDD-induced *Cyp1a1*. The benefits of IAld in ameliorating colitis and inflammation, inhibiting fungal infection, and promoting intestinal barrier regeneration and proliferation have also been demonstrated to be AhR-dependent using $AhR^{-/-}$ mice or synthetic inhibitors[45,125,130,131].

Our work, as well as work from other labs, have shown that indole and other MDTMs show AhR ligand activity in epithelial cells and hepatocytes[41,44,61,99,130]. However, not much is known about the AhR ligand activity of MDTMs in other immune cells. Even less is known about pathways or transcription factors other than the AhR that may play a role in MDTM-mediated signaling. The effects of IAld on graft-vs-host disease (GVHD) have been investigated in mice receiving mismatched allogeneic bone

marrow transplantation. Administration of IAld was protective of GVHD induced mortality and attenuated multiple markers of disease related to gut inflammation[188]. Transcriptome profiling and subsequent experiments with interferon-alpha/beta receptor knockout mice demonstrate these protective effects were mediated by type I interferons. Interestingly, transcriptome analysis did not reveal changes in AhR signaling in this disease model, suggesting further context dependency of MDTM signaling. Another MDTM, indole-3-acetic acid (IAA), attenuated LPS stimulated cytokine production and gene expression in macrophages *in vitro*[46]. Interestingly, chemical antagonism of the AhR did not significantly alter the anti-inflammatory effects of IAA, suggesting an AhR independent mechanism[46]. Conversely, a competitive inhibitor of heme oxygenase 1 (HO-1), tin protoporphyrin IX (SnPP), impeded the anti-inflammatory effects of IAA. Expression of the gene encoding HO-1, *Hmox1*, was also induced with IAA incubation, suggesting an HO-1 dependent mechanism in macrophages. *Hmox1* is known to be transcriptionally activated by nuclear factor erythroid 2-related factor 2 (NRF2), making this a novel non-AhR mechanistic target of MDTMs[189].

2.5. NRF2

The nuclear factor erythroid 2-related factor 2 (NRF2) transcription factor and its orthologues are expressed across multiple kingdoms of life and evolved as a master regulator of oxidative stress, electrophilic response, xenobiotic metabolism, and immune regulation[190]. NRF2 has long been an attractive target for therapeutic intervention. Treatments for psoriasis and multiple sclerosis using dimethyl fumarate, an NRF2

activator, have been approved for decades, with many other NRF2 drugs for other diseases in phase III trials[191-193]. At homeostasis, NRF2 activity is limited by Kelch-like ECH-associated protein 1 (KEAP1), which acts as an adaptor protein for ubiquitin ligases that modify NRF2 to signal its proteasomal degradation[194]. Critical cysteine residues of KEAP1 are susceptible to covalent modification by electrophilic compounds, stimulating conformational changes resulting in dissociation from NRF2 (**Figure 2.4**)[195]. These interactions facilitate sensitive response to compounds like reactive oxygen and nitrogen species and underlie the function of NRF2 in redox regulation[195]. Upon liberation from KEAP1, NRF2 translocates to the nucleus and forms heterodimers with small musculoaponeurotic fibrosarcoma (sMAF) proteins. This complex binds antioxidant response elements in the promoter region of target genes, primarily those involved in the glutathione and thioredoxin antioxidant response systems, heme metabolism, xenobiotic metabolism, and in the generation of NADPH, an obligate co-factor for many of the enzymatic reactions in these systems[196]. Typical target genes of NRF2 include *Hmox1*, *Nqo1*, the system x_c^- cystine-glutamate transporter (*Slc7a11*), and the genes encoding the catalytic and modifier subunits of glutamate cysteine ligase (GCL), *Gclc* and *Gclm,* respectively.

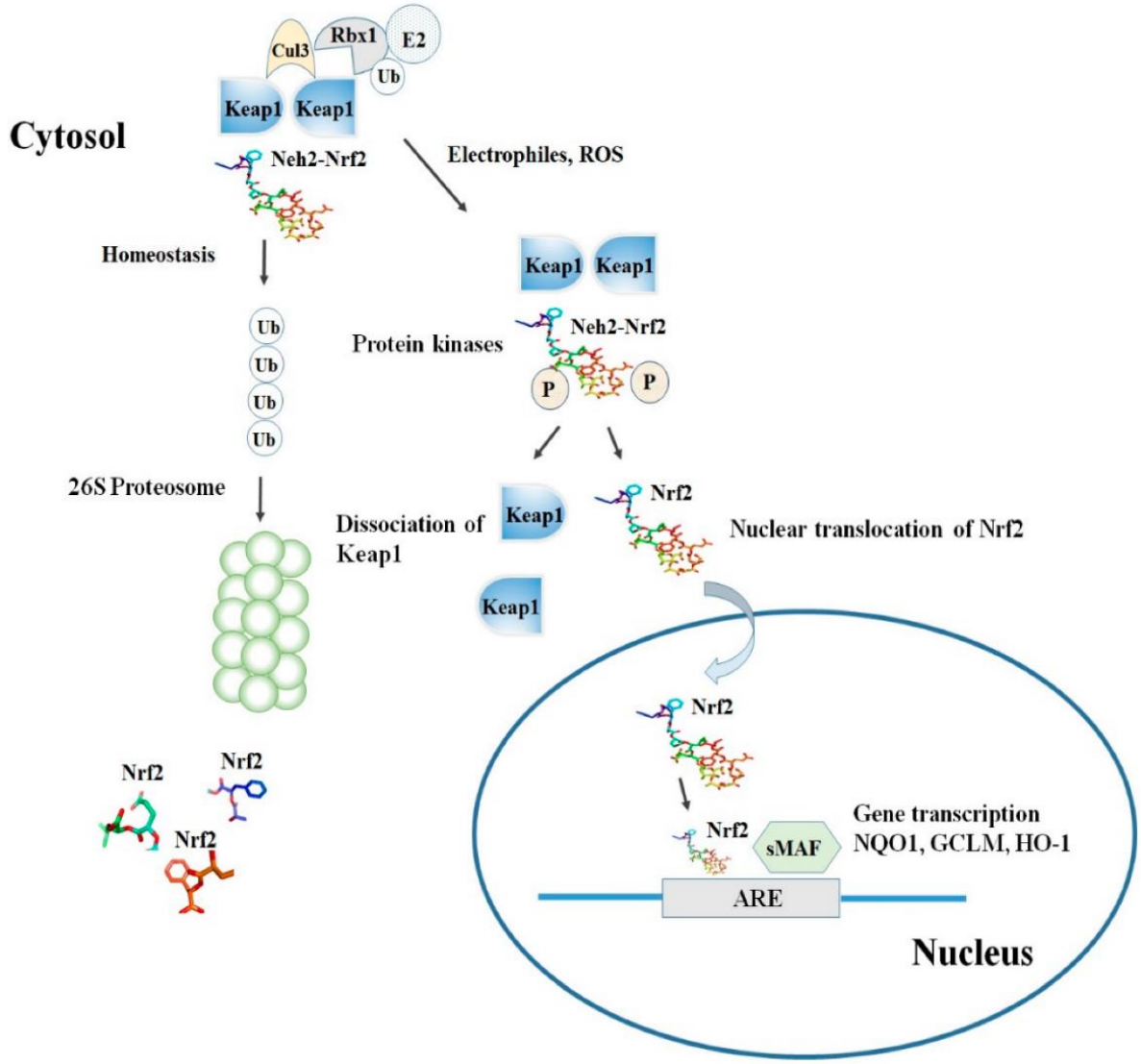


Figure 2.4. NRF2 transcription factor regulation. A cytosolic complex of Kelch-like ECH-associated protein 1 (KEAP1), cullin 3 (CUL3), and ring box protein 1 (RBX1) facilitate ubiquitination of NRF2, resulting in proteosomal degradation. Activation of NRF2 signaling through various signals, including electrophilic and reactive oxygen stressors, stimulate conformational changes in critical binding regions resulting in the release of NRF2 from the ubiquitinating complex, allowing for nuclear translocation. Subsequent dimerization with sMAF proteins results in targeted binding to antioxidant response elements (AREs) and upregulation of NRF2 target genes. Modified from Saha *et al*[195].

NRF2 and inflammatory signaling can intersect directly because of these canonical antioxidant responses, as oxidative stress can drive chronic inflammation. It

has also been proposed that NRF2 and nuclear factor kappa-light-chain-enhancer of activated B (NF-κB), a major transcriptional complex involved in inflammatory signaling, compete for binding to the CREB binding protein (CBP). In the nucleus, both NRF2 and NF-κB utilize CBP as a necessary coactivator for transcriptional activation[197]. Furthermore, NRF2 has been shown to bind near the *Il6* and *Il1b* gene promoter regions in macrophages, inhibiting their transcription independent of redox signaling[198]. While NRF2 binding near canonical targets, i.e. *Hmox1* and *Nqo1*, was consistently high in both macrophages and hepatocytes, binding near the afore-mentioned pro-inflammatory interleukins was not observed in liver cells, demonstrating cell-context dependent function[198]. Further connecting multiple axes of signaling, the MDTM indole-3-acetic acid inhibited LPS-stimulated cytokine production in macrophages through a *Hmox1*-dependent mechanism.

2.6. microRNAs

Regulation of protein levels in cells is facilitated by multiple mechanisms. These include transcription of mRNA, stability of mRNA, protein degradation, and post-translational modification of proteins. An emerging evolutionarily conserved mechanism of regulation across multiple kingdoms of life is microRNA-mediated gene silencing, which can affect both mRNA stability and repression of translation[56,105,177]. Considering that greater than 60% of protein coding genes in humans contain at least one conserved miRNA binding site, it is not surprising that miRNAs have been found to be involved in a wide range of diseases, including cancer, cardiovascular disease, diabetes, and

IBD[199,200]. The dysregulation of microRNA (miRNA) processing machinery has been associated with multiple types of cancer, along with the upregulation of pro-oncogenic miRNAs, and down-regulation of tumor suppressive miRNAs[201]. In Crohn's disease, miRNA signaling has been proposed to explain the identification of a susceptibility loci involving a synonymous single-nucleotide polymorphism. While a polymorphism of this nature in immunity related GTPase M (IRGM) does not alter the amino acid sequence, it was found to impair binding of miR-196, resulting impaired autophagic response to invasive *E. coli* infection in HEK293 cells[200].

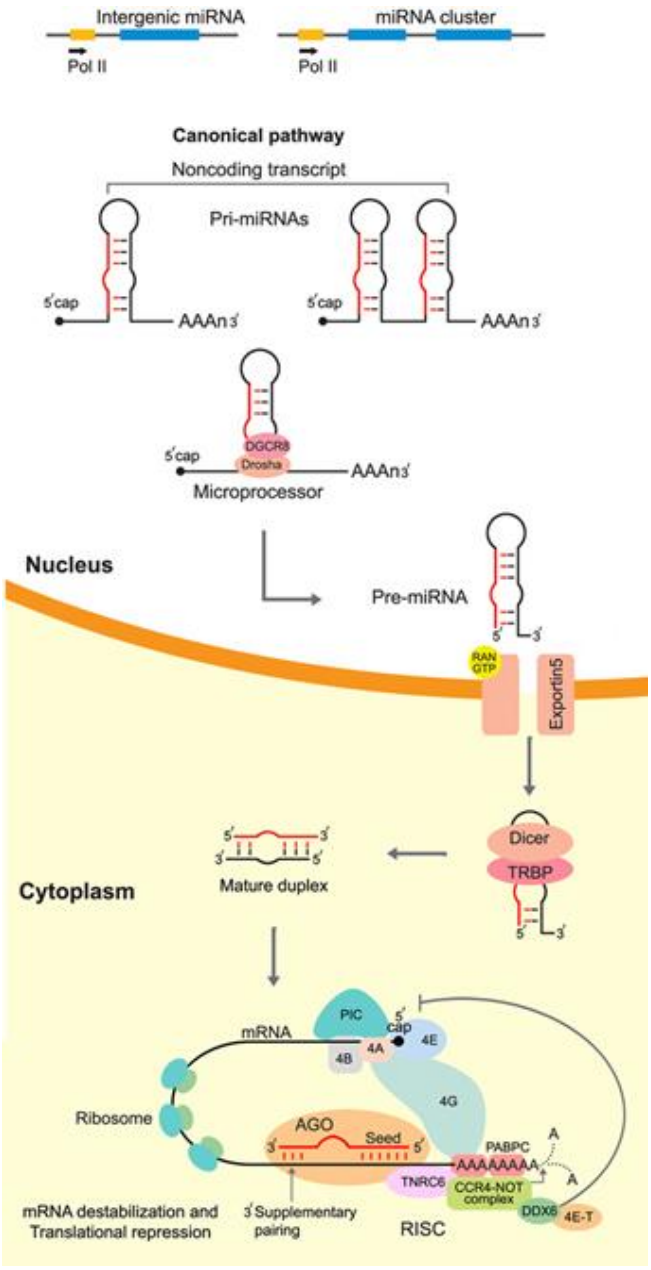


Figure 2.5. MicroRNA biogenesis and function. RNA polymerase II transcribes miRNA sequences into primary miRNAs (pri-MRNAs). Within the nucleus, pri-MRNAs are cleaved by the Microprocessor complex, composed of Drosha and DiGeorge syndrome critical region gene 8 (DGCR8). The resulting precursor RNAs (pre-miRNAs) are transported to the cytoplasm by Exportin 5, where a complex of Dicer and TAR RNA binding protein (TRBP) trim the hairpin structure, resulting in mature double stranded miRNA. The RNA-induced silencing complex (RISC) facilitates silencing of targeted mRNAs based on the guide strand of the mature miRNA duplex. Modified from Saliminejad *et al*[202].

In animal hosts, miRNA genes primarily reside in introns of both coding and non-coding transcripts, but may also exist in exons or intergenic regions with independent promoter sequences[203,204]. When embedded within a coding gene, miRNA transcription can be under the control of the same promoter regions of the parent gene, resulting in co-expression dynamics[201]. Upon transcription, a primary miRNA containing an internal hairpin structure is synthesized by RNA polymerase II (**Figure 2.5**)[202]. The primary miRNA is then cleaved by the Microprocessor complex and Dicer, resulting in a small double-stranded RNA ~22 nucleotides long. This small RNA is then loaded in an Argonaute protein where the RNA is unwound, and a guide strand is chosen. This mature miRNA and the Argonaute protein form a RNA-induced silencing complex (RISC) that is capable of targeted destabilization or repression of mRNA guided by the binding affinity of the miRNA. The RISC typically binds the 3' untranslated region of mRNAs, inhibiting subsequent translation or signaling the degradation for the degradation of the bound mRNA[202].

Work in the HeLa human epithelial cell line has suggested that miRNA-mediated regulation of protein production occurs primarily through decreasing mRNA levels, with 11-16% of the reduction in protein output being attributed to translational repression, though mRNA destabilization is often preceded by translational repression[205]. However, the relative contributions of each modality are still not fully understood and it is likely that preferred mechanisms are mRNA-target and cell context dependent[206]. This is evident in reports of miRNA-mediated protein regulation that result in no alteration in

measured mRNA levels[207,208]. Furthermore, knockout of the DDX6 gene in embryonic stem cells selectively increased the translation of miRNA targets without altering mRNA stability, demonstrating translational repression by miRNA alone is sufficient to alter protein production[209].

Multiple miRNA-based therapeutics for treatment of hepatitis C or type 2 diabetes in conjunction with non-alcoholic fatty liver disease have entered phase II clinical trials[210]. An antisense oligonucleotide treatment, Miravirsen, capable of sequestering miR-122 was shown to reduce hepatitis C viral RNA in a dose dependent manner and specifically decreased miR-122 levels in circulation[211,212]. A similar anti-miR-122 treatment, RG-101, also reduced hepatitis C viral RNA levels and was found to restore natural killer cell population frequencies in patients with chronic hepatitis C infection[210,213]. Antagonism of the related miRNAs miR-103 and miR-107 in adipocytes lead to improved insulin sensitivity and glucose uptake in a murine model of type 2 diabetes and obesity[214]. A phase II clinical for an anti-miR-103/107 therapeutic, AZD4076, was initiated, but ultimately, development of the therapeutic was halted. Interestingly, subsequent work found overexpression of miR-103 in hepatocytes to alleviate NAFLD in mice, demonstrating cell-context dependency in the benefit of miR signaling in disease[215]. Overall, the current literature on miRNAs suggests their ubiquitous involvement in human health and present a model of gene regulation that may explain complex phenotypes and are a promising target for therapeutic development.

3. NRF2-DEPENDENT ANTI-INFLAMMATORY ACTIVITY OF INDOLE VIA CELL SURFACE INTERACTION

3.1. Overview

The anti-inflammatory effects of indole and other microbially-derived tryptophan metabolites have been well documented; however, the precise mechanisms of action remain largely unknown. While the aryl hydrocarbon receptor has been implicated as a potential cytoplasmic receptor for indole and indole derivatives, we have found the anti-inflammatory effects of indole to be AhR-independent in macrophages, prompting us to investigate the involvement of other receptors for microbially-derived tryptophan metabolites. To explore the possibility of surface-receptor mediated signaling, we developed an indole-bovine serum albumin conjugate, I3B, which would primarily engage cell surface receptor(s). Reduction of LPS stimulated TNF production in RAW 264.7 macrophages with 10 μM I3B was similar to that observed with indole at 500 μM, suggesting potent cell surface mediated effects. Transcriptome profiling of I3B-treated RAW264.7 macrophages revealed that the LPS-induced pro-inflammatory response was blunted in cells exposed to I3B. The gene expression data show signatures of NRF2 activation, with the upregulation of NRF2 target genes *Hmox1, Slc7a11, Nqo1, and Gclm* (17.69, 7.61, 7.2, and 4.86-fold, respectively). I3B-primed bone marrow-derived macrophages (BMM) from $NRF2^{-/-}$ mice showed reduced upregulation of *Hmox1* and higher levels of cytokine production in response to LPS stimulation relative to wild-type

BMM cells. Our results suggest I3B signals through a surface-receptor in a NRF2-dependent manner to reduce inflammation in murine macrophages.

3.2. Introduction

Indole is a tryptophan metabolite that can by produced by microbial symbionts via the enzyme tryptophanase[121]. Analysis of human gut metagenomic data suggest numerous phyla of bacteria, including *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, that are present in the human gastrointestinal tract have genes encoding TnaA (or similar enzymes) and hence are capable of producing indole.[216]. Studies have found median fecal indole concentrations in human fecal samples to be approximately in the low millimolar range[57-60], with one study reporting values ranging from 0.3 to 6.64 mM in healthy adults[58]. Several *in vitro* and *in vivo* studies have demonstrated a role for indole in modulating inflammation in multiple models, ranging from suppressing diet-induced NAFLD and liver inflammation[48,49], to protecting against dextran sodium sulfate induced colitis and promoting intestinal epithelial function[39,40], to reducing central nervous system inflammation in a murine model of multiple sclerosis[42]. In addition to indole, other microbially derived tryptophan metabolites (MDTMs), including indole-3-aldehyde (IAld), indoxyl-3-sulfate, indole-3-propionic acid, indole-3-acetate, indole-3-ethanol, and indole-3-pyruvate, have also demonstrated similar protective effects in various model systems[42,44,45,125,188,217,218].

Several studies have shown that indole and other MDTMs signal through the cytosolic aryl hydrocarbon receptor (AhR) pathway in different cell types, including

epithelial cells and hepatocytes[42,44,45,61,99,125,218]. However, AhR-dependent effects can be cell-context dependent and it is unclear whether these molecules can potentially engage other receptors with varied levels of activity across different cell types[61]. Furthermore, while there are some reports on the effects of MDTMs on macrophages, the AhR-dependent activity of these compounds in macrophages is yet to be fully understood[44,49]. As most investigations into the mechanistic signaling of MDTMs has been focused on the AhR, knowledge on the interactions of MDTMs with other receptors, such as cell surface receptors, is limited.

In this study, we investigated the effect of indole on macrophages and characterized the AhR dependence using an AhR antagonist and $AhR^{-/-}$ mice. We further explored the potential for indole to engage cell surface receptors, as opposed to cytosolic receptors like the AhR, by synthesizing a membrane impermeant indole-bovine serum albumin conjugate (I3B). Our results demonstrate that the potent anti-inflammatory effects of I3B are AhR-independent and partially dependent on the nuclear factor erythroid 2-related factor 2 (NRF2) signaling pathway.

3.3. Results

3.3.1. Indole modulates TNF production in RAW264.7 macrophages independent of the AhR

The effect of indole on LPS-stimulated TNF accumulation in the murine macrophage cell line, RAW264.7, was determined utilizing intracellular cytokine staining (ICS) flow cytometry. Cells pre-incubated with indole for 4 h followed by 6 h of

co-stimulation with LPS significantly attenuated TNF accumulation relative to vehicle control in a dose-dependent manner, as determined by both MFI and percentage of TNF-positive live cells (**Figure 3.1**). At concentrations of 0.5 and 1 mM, indole reduced the proportion of TNF-positive cells by roughly 26% and 43%, respectively. The decrease in TNF accumulation was also observed in primary bone-marrow derived macrophages (BMM) from both WT and $AhR^{-/-}$ mice, albeit with reduced potency (**Figure 3.2**). Incubation with 1 mM indole resulted in roughly 16% and 27% reduction in TNF-positive cells in WT and $AhR^{-/-}$ BMM, respectively. No statistically significant differences were observed between the WT and $AhR^{-/-}$ conditions, suggesting indole inhibits LPS-stimulated TNF accumulation in macrophages independent of the AhR.

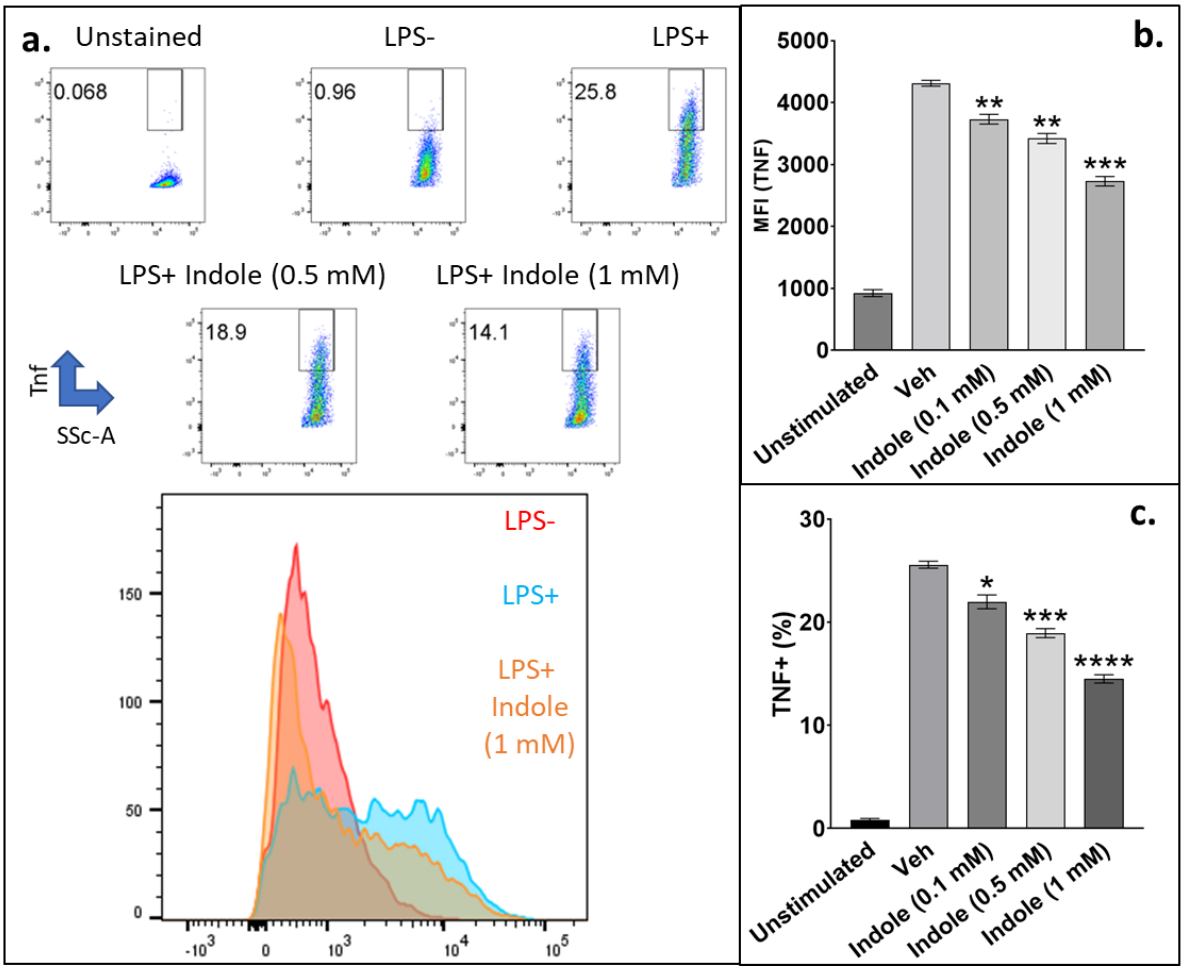


Figure 3.1. Indole inhibits LPS stimulated TNF accumulation. RAW264.7 macrophages were pre-incubated with indole or vehicle control for 4 h prior to co-stimulation with 250 ng/mL LPS for 6 h. (**a**) Representative gating strategy, scatter plots, and histograms of cells analyzed with ICS flow cytometry. Quantification of TNF protein through (**b**) mean fluorescence intensity (arbitrary units) and (**c**) percent TNF-positive cells. Bars represent mean values from three or four independent cell cultures and error bars represent ± S.E.M. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

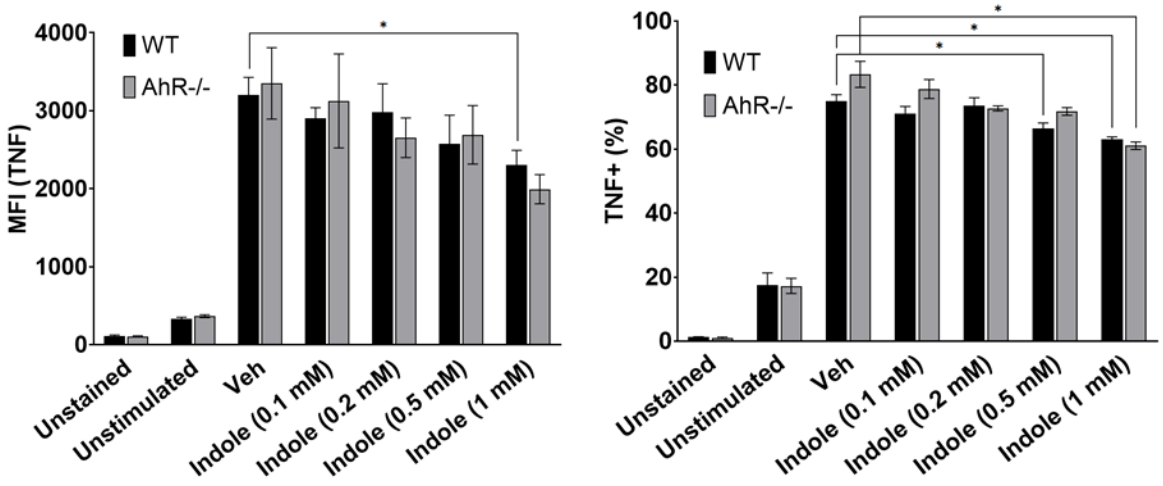


Figure 3.2. Indole inhibits LPS stimulated TNF accumulation independent of the AhR. BMM from WT or AhR$^{-/-}$ mice were pre-incubated with indole or vehicle control for 4 h prior to co-stimulation with 20 ng/mL LPS for 6 h. Cells were stained for TNF protein and analyzed using ICS flow cytometry. Quantification of TNF protein through (**a**) mean fluorescence intensity (arbitrary units) and (**b**) percent TNF-positive cells. Gating strategies are represented by those shown in **Figure 3.1a**. Bars represent mean values from three or four independent cell cultures and error bars represent ± S.E.M. * $p < 0.05$.

3.3.2. BSA conjugation potentiates the anti-inflammatory properties of indole

To further investigate the AhR-independent, cell surface localized effects of indole, we conjugated indole-3-aldehyde (IAld) to bovine serum albumin (BSA) using the crosslinker, N-κ-maleimidoundecanoic acid hydrazide (KMUH), resulting in a 1:1:1 stoichiometry between indole moieties, KMUH, and BSA (**Figure 3.3a**). The end-product, I3B, comprises of an indole moiety bound to one end of an 11-carbon aliphatic spacer via a hydrazone bond, with a thioether bond linking BSA at the opposing end of the spacer. The predicted mass shift of I3B relative to BSA is 423 Da, and MALDI-TOF mass spectrometry analysis showed an observed mass shift of 449 Da (**Figure 3.3b**).

We posited that the uptake of indole is inhibited due to steric hindrance from the conjugated 66 kDa BSA. A construct made with green fluorescent protein instead of BSA using similar chemistry showed absence of intracellular fluorescence even after 16 h (**Figure 3.3c**), supporting the hypothesis that indole conjugated to a larger protein was excluded from RAW 264.7 macrophages. Pre-incubation with 10 μM I3B attenuated the LPS stimulated TNF accumulation in RAW264.7 macrophages to a level similar to that observed with 1 mM free indole, while higher concentrations of 50 and 100 μM I3B reduced TNF accumulation to levels lower than the unstimulated controls (**Figure 3.4**). Pre-incubation with free indole, IAld, or BSA (alone or in combination) at equivalent concentrations did not recapitulate the potent effects of I3B, suggesting this potentiation results from increased indole engagement with the cell surface.

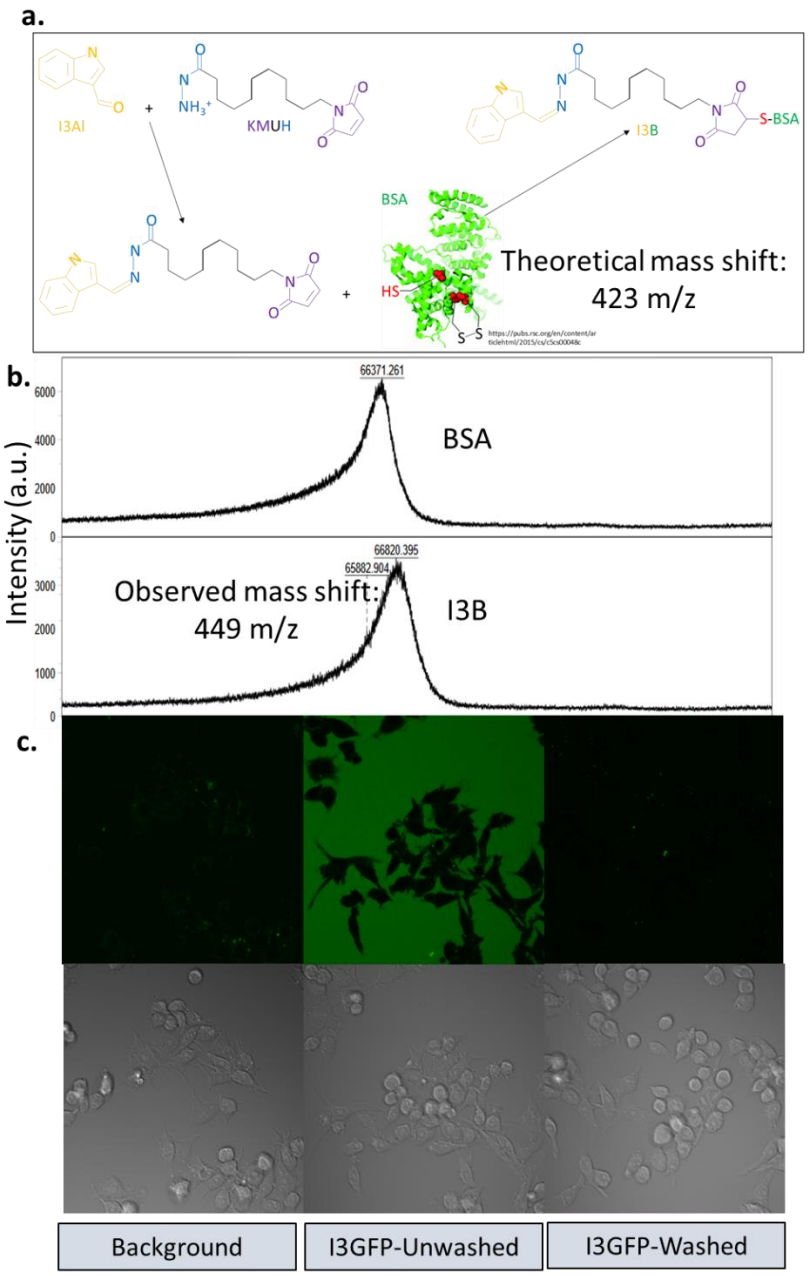


Figure 3.3. Synthesis of I3B. (a) IAld was reacted with KMUH in DMSO at a 5:1 molar ratio. The KMUH-IAld reaction product was reacted with BSA at a 5:1 molar ratio of KMUH equivalents to BSA. Unreacted reagents were removed with a desalting column followed by concentration of product and buffer exchange through a centrifugal filter. Desalted I3B without buffer exchange were analyzed for mass shift **(b)** relative to pure BSA using MALDI-TOF mass spectrometry. A similar synthesis was carried out with GFP replacing BSA **(c)**, with the addition of Traut's reagent to functionalize lysine groups for reaction with KMUH.

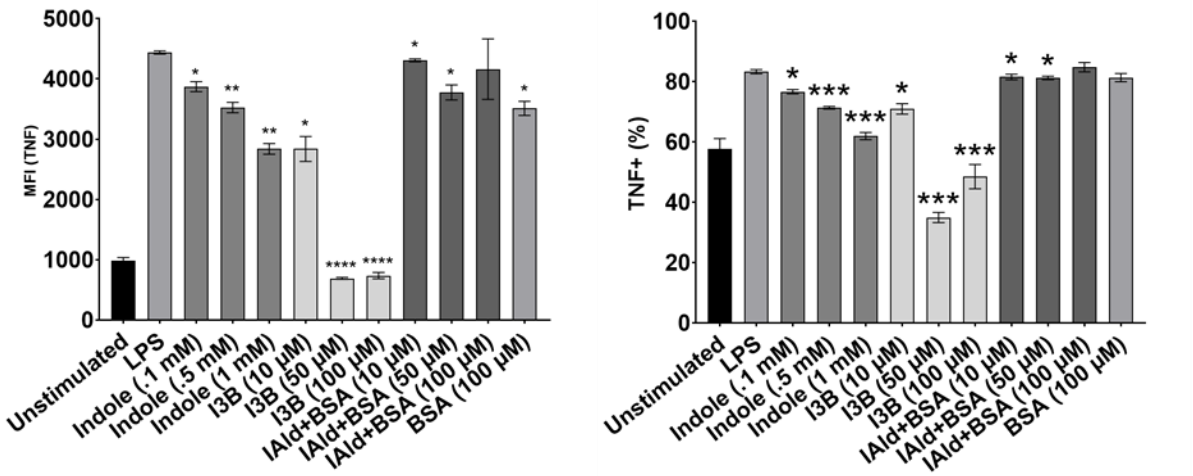


Figure 3.4. Effects of I3B on LPS stimulated TNF accumulation. RAW264.7 macrophages were pre-incubated with compounds of interest for 4 h followed by 6 h of 250 ng/mL LPS stimulation. Cells were fixed and stained for TNF for ICS flow cytometry analysis. Gating strategies are represented by those shown in **Figure 3.1a**. Bars represent mean values from three or four independent cell cultures and error bars represent ± S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To further investigate the effects of I3B on modulation of inflammatory cytokine production, RAW264.7 macrophages were pre-incubated with I3B for 4 h followed by LPS stimulation for 24 h. Cell culture supernatants were analyzed via the LEGENDPlex^{TM} Mouse Inflammation Panel (**Figure 3.5**). Pre-incubation with 100 µM I3B significantly reduced secretion of TNF, recapitulating the ICS flow cytometry results. Unstimulated RAW264.7 cells did not secrete detectible levels of IL-6, IFN-β, and MCP-1. While LPS stimulation increased the concentration of these pro-inflammatory cytokines to 225, 124, and 9.4 pg/mL, respectively, I3B pre-treatment reduced the concentration of these cytokines to below the limit of detection. Intriguingly, incubation with I3B alone stimulated a five-fold increase in the secretion of anti-inflammatory IL-10 (from 122 pg/mL to 610 pg/mL). These results suggest I3B pre-

incubation blunts RAW 264.7 macrophage response to LPS consistent with an anti-inflammatory response.

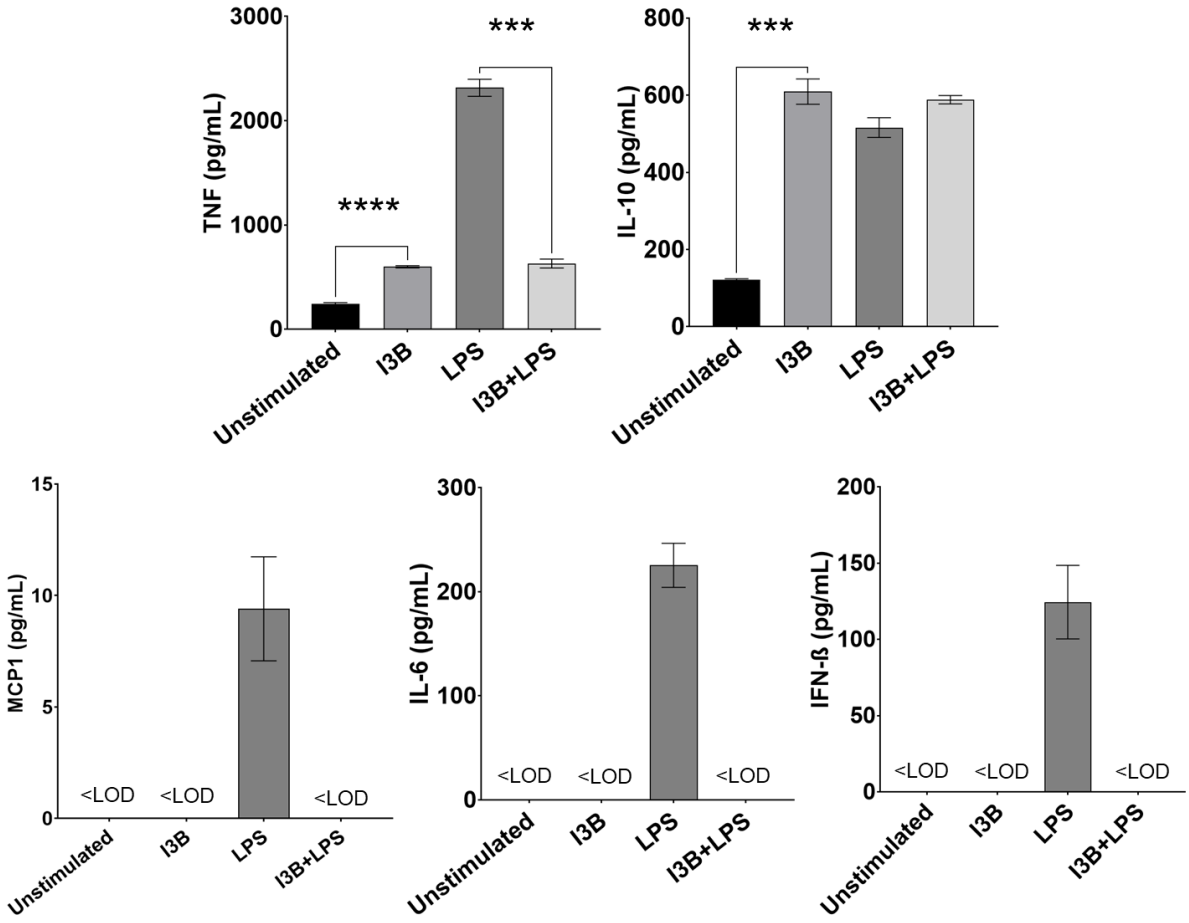


Figure 3.5. Anti-inflammatory cytokine secretion profile of I3B-primed macrophages. RAW264.7 cells were pre-incubated with I3B for 4 h then co-stimulated with LPS for 24 h. Cell culture supernatants were analyzed with the LegendPlex™ multiplexed antibody bead-based assay to quantify cytokine secretion. Results represent data from three independent cell cultures with two technical replicates for each culture supernatant. Error bars represent ± S.E.M. *** $p < 0.001$, **** $p < 0.0001$, <LOD: cytokines were not observed above the limit of detection.

3.3.3. Effects of I3B in macrophages are independent of the AhR

The involvement of AhR in mediating indole, IAld, and other MDTMs signaling is well documented[41,45,61,125,219]. Interestingly, the AhR is a cytosolic receptor that is not expressed at the cell surface membrane, leaving direct engagement of the AhR by I3B an unfeasible occurrence. Furthermore, we found no quantifiable levels of either AhR or *Cyp1a1* gene expression in RAW264.7 macrophages upon stimulation with AhR ligands, TCDD and diindolylmethane, despite previous reports[220]. However, the AhR is one of few receptors hypothesized to play a major role in indole signaling, leading us to further probe its potential role in indole and I3B signaling in macrophages. RAW264.7 cells were pre-incubated with 20 μM of AhR inhibitor, CH-223191, for 14 h, before co-incubation with indole or I3B for 4 h, and LPS stimulation for the final 6 h before analysis via ICS flow cytometry. CH-223191 alone increased accumulation of TNF and percentage of cells that were TNF^+ (**Figure 3.6a, c**). After normalization to the stimulated control, pre-incubation with CH-223191 had no significant effect on TNF accumulation or percent TNF^+ for either indole or I3B conditions (**Figure 3.6b, d**). Furthermore, BMM were cultured from wild-type (WT) and $AhR^{-/-}$ mice and were incubated with I3B and stimulated with LPS as previously described. ICS flow cytometry showed that both indole and I3B attenuated LPS-stimulated TNF accumulation, in both the WT BMM and $AhR^{-/-}$ BMM (**Figure 3.6e, f**). Comparisons between WT and $AhR^{-/-}$ BMM were non-significant, suggesting indole and I3B function through an AhR-independent mechanism to inhibition of LPS stimulation in macrophages.

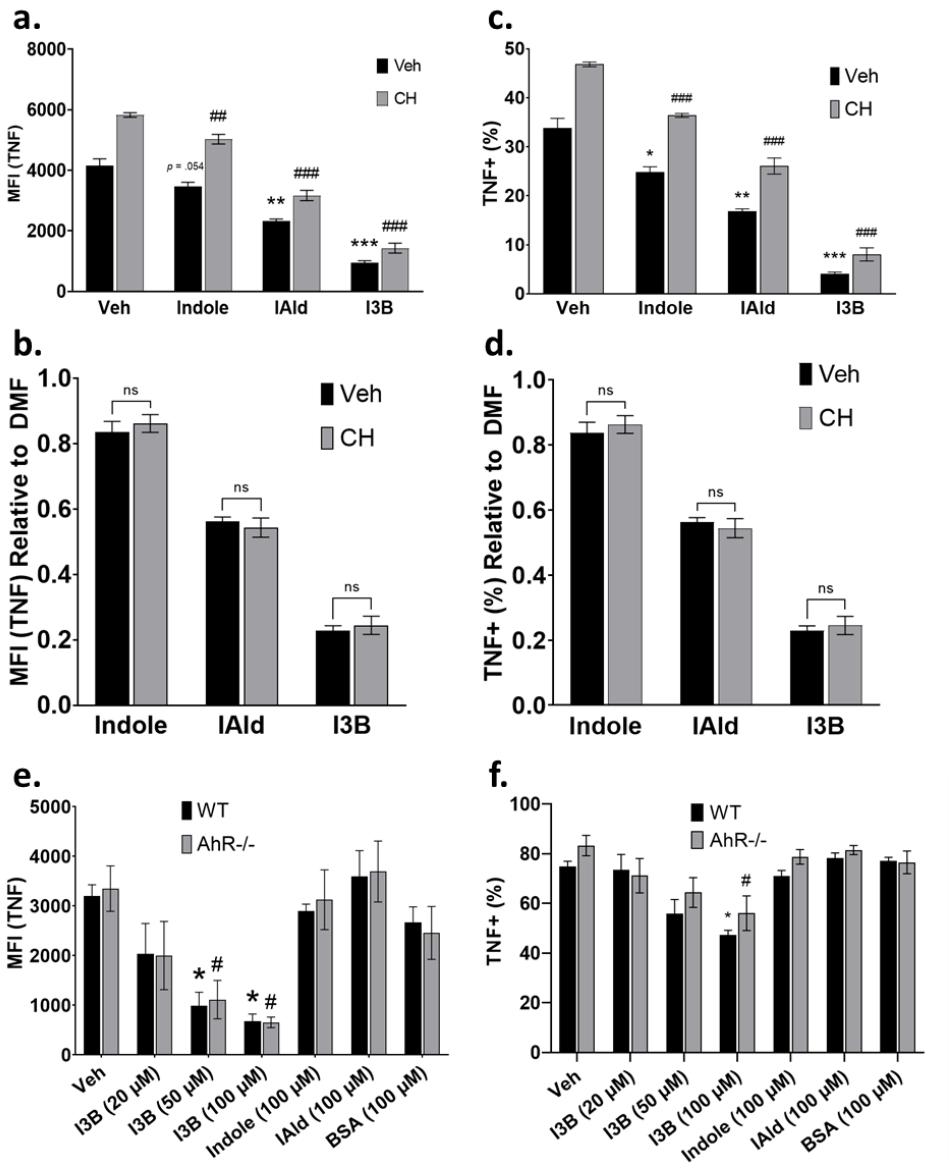


Figure 3.6. Effects of I3B on macrophages is AhR independent. RAW264.7 macrophages were pre-incubated with CH-223191 (CH) for 14 h followed by co-incubation with I3B, indole, IAld, or vehicle control for 4 h. Cells were then co-stimulated with 250 ng/mL LPS for 6 additional hours. Absolute **(a, c)** and relative **(b, d)** measures of TNF accumulation from ICS flow cytometry are shown. **(e, f)** BMM from WT and AhR$^{-/-}$ mice were incubated with compounds of interest for 4 h followed by 6 h of 20 ng/mL LPS stimulation and analyzed with ICS flow cytometry. Gating strategies are represented by those shown in **Figure 3.1a**. Error bars represent ± S.E.M. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, relative to vehicle control for compounds of interest. #, $p < .05$ relative to AhR$^{-/-}$ vehicle control, ## $p < 0.01$, ### $p < 0.001$ relative to vehicle control for CH.

3.3.4. Transcriptional analysis of I3B-treated macrophages

We utilized whole-transcriptome profiling to obtain a global profile of changes in gene expression in RAW264.7 macrophages upon I3B stimulation. Using a two-factor factorial design, RAW264.7 macrophages were incubated with or without 50 μM I3B for 4 h, followed by incubation with or without LPS for 1 h. RNA sequencing was used for expression profiling followed by gene set enrichment analysis (GSEA). Multidimensional scaling revealed three distinct transcriptomic clusters, consisting of the unstimulated control, LPS-stimulated control, and both I3B conditions (I3B with and without LPS) (**Figure 3.7a**). As expected, LPS stimulated upregulation of inflammatory cytokines, including *Cxcl2, Cxcl10, Egr2, Tnfaip3, Ccrl2, Ifnb1, and Tnf* (88.6, 40.3, 21.6, 19.6, 19.6, 12.3, and 10.1-fold, respectively) (**Table 3.1**). However, when cells were preconditioned with I3B, subsequent stimulation by LPS did not result in a significant induction in expression of these cytokines (**Figure 3.7b**),

I3B alone upregulated the expression of 334 genes and downregulated that of 77 genes (fold change ≥ 2, $p < 0.05$) relative to the unstimulated control, and this set of genes was distinct from that altered by LPS (**Table 3.2**). The list of genes up-regulated by I3B included *Sel1l3, Cox6a2, Slc40a1,* and *Hmox1* (34.5, 23.2, 20.4, and 17.7-fold, respectively). Stimulation of I3B pre-conditioned cells with LPS largely did not alter the transcriptome of this set of genes (**Figure 3.7c**). Patterns of gene expression in *Tnf, Hmox1,* and *Cxcl10,* were validated using qRT-PCR (**Figure 3.7d**).

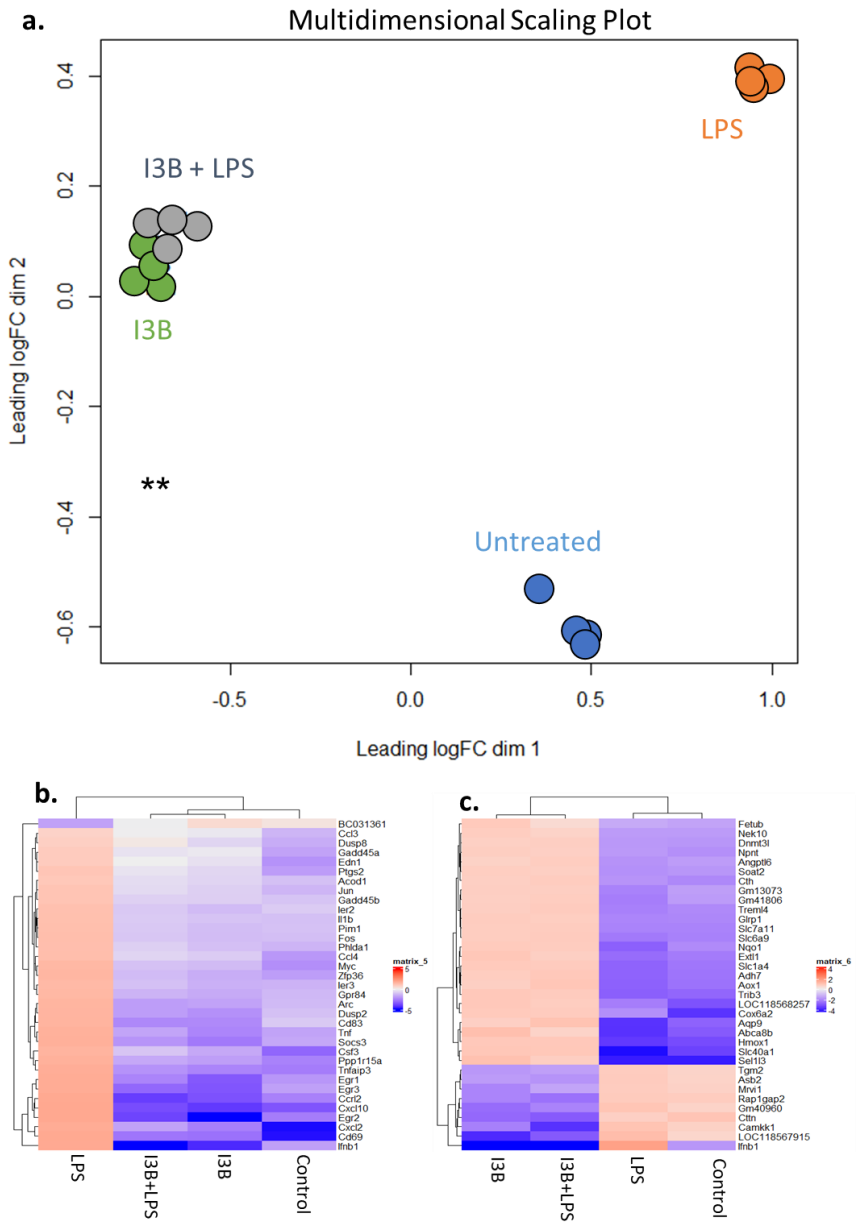


Figure 3.7. Transcriptome profiling of I3B treated RAW264.7 macrophages. Cells were pre-incubated with I3B for 4 h followed by 1 h of 250 ng/mL LPS stimulation before mRNA isolation. mRNA libraries were prepared for paired-end RNA-sequencing with differential gene expression analysis done in EdgeR. **(a)** Multidimensional scatterplot representing the four treatment conditions with four independent replicates. Clustered heat maps of the top 35 most differentially regulated genes by **(b)** LPS stimulation and by **(c)** I3B treatment alone. Differential gene expression were row normalized and $\log_2$ transformed. **(d)** qRT-PCR validation of select differentially regulated genes (n = 3). * $p < 0.05$, ** $p < 0.01$.

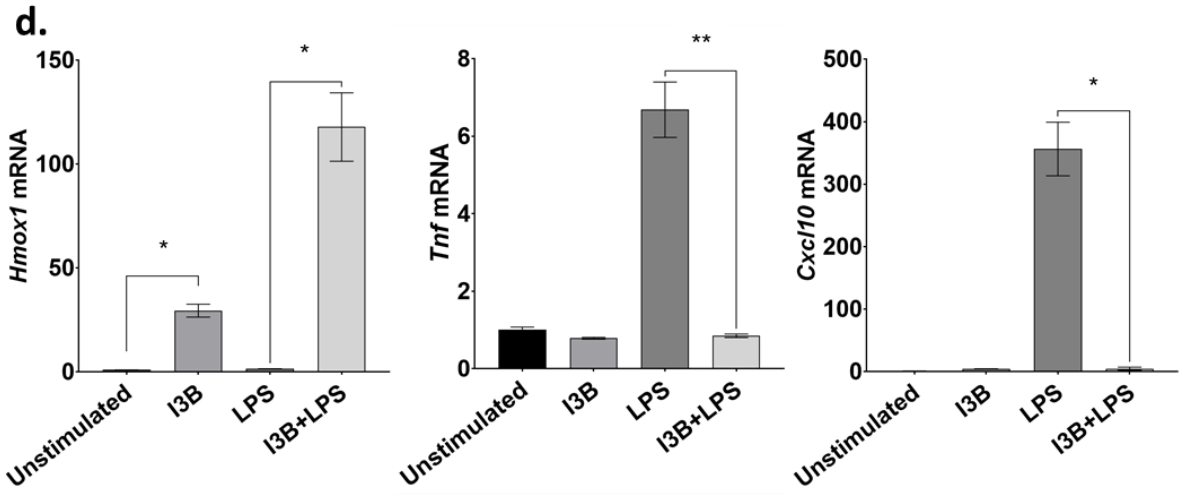


Figure 3.8. Continued.

3.3.4.1. Gene set enrichment analysis

Gene Set Enrichment Analysis (GSEA) was used to further analyze the transcriptome data. Two gene set collections – hallmark and Wikipathway – were used for the comparative analysis. GSEA showed that incubation with I3B enriched 18 of the 49 gene sets in the hallmark gene set collection (nominal $p < 0.01$, FDR < 0.2). Gene sets with the highest normalized enrichment score (NES) were unfolded protein response (2.52), P53 pathway (2.50), reactive oxygen species pathway (2.38), xenobiotic metabolism (2.35), MTORC1 signaling (2.33), and heme metabolism (2.19) (**Figure 3.8**). Analysis of the Wikipathway collection revealed enrichment in 71 of the 337 gene sets (nominal $p < .01$, FDR < .2). Of particular interest was the NRF2-ARE (nuclear factor, erythroid 2-like 2-antioxidant response element) regulation gene set (NES = 2.48, rank 5), that contains multiple genes for phase II metabolizing enzymes upregulated by

I3B, such as *Hmox1*, *Slc7a11*, *Nqo1*, and *Gclm* (17.69, 7.61, 7.20, and 4.86-fold upregulation, respectively).

Table 3.1 Top differentially regulated genes in LPS stimulated RAW264.7 cells.

	Fold Change	*p*-value
Cd69	90.6	2.7E-18
Cxcl2	88.6	7.1E-41
Cxcl10	40.3	9.4E-38
Csf3	24.7	1.4E-21
Egr2	21.6	1.5E-31
Tnfaip3	19.6	3.3E-43
Ccrl2	19.6	1.3E-27
Ppp1r15a	16.8	1.8E-38
Egr1	13.4	8.1E-42
Ifnb1	12.3	2.8E-13
Myc	12.2	9.7E-16
Egr3	11.6	3.3E-35
Tnf	10.1	1.5E-41
Zfp36	10.0	5.0E-43
Socs3	9.7	1.3E-37
Ccl4	9.4	1.7E-35
Edn1	8.1	1.1E-12

Table 3.2 Top differentially regulated genes in I3B treated RAW264.7 cells

	Fold Change	*p*-value
Sel1l3	34.48	2.8E-09
Cox6a2	23.22	1.1E-08
LOC118567915	0.05	1.2E-12
Slc40a1	20.37	3.3E-25
Hmox1	17.69	7.1E-28
Abca8b	16.92	1.0E-07
LOC118568257	16.16	1.5E-06
Cttn	0.08	1.0E-08
Trib3	12.36	2.4E-26
Aqp9	11.54	8.7E-18
Slc1a4	9.68	1.8E-28
Gm40960	0.10	1.2E-11
Adh7	9.53	2.7E-14
Extl1	9.45	1.9E-12
Aox1	9.37	2.2E-12
Slc6a9	7.93	6.5E-28
Slc7a11	7.61	3.0E-34

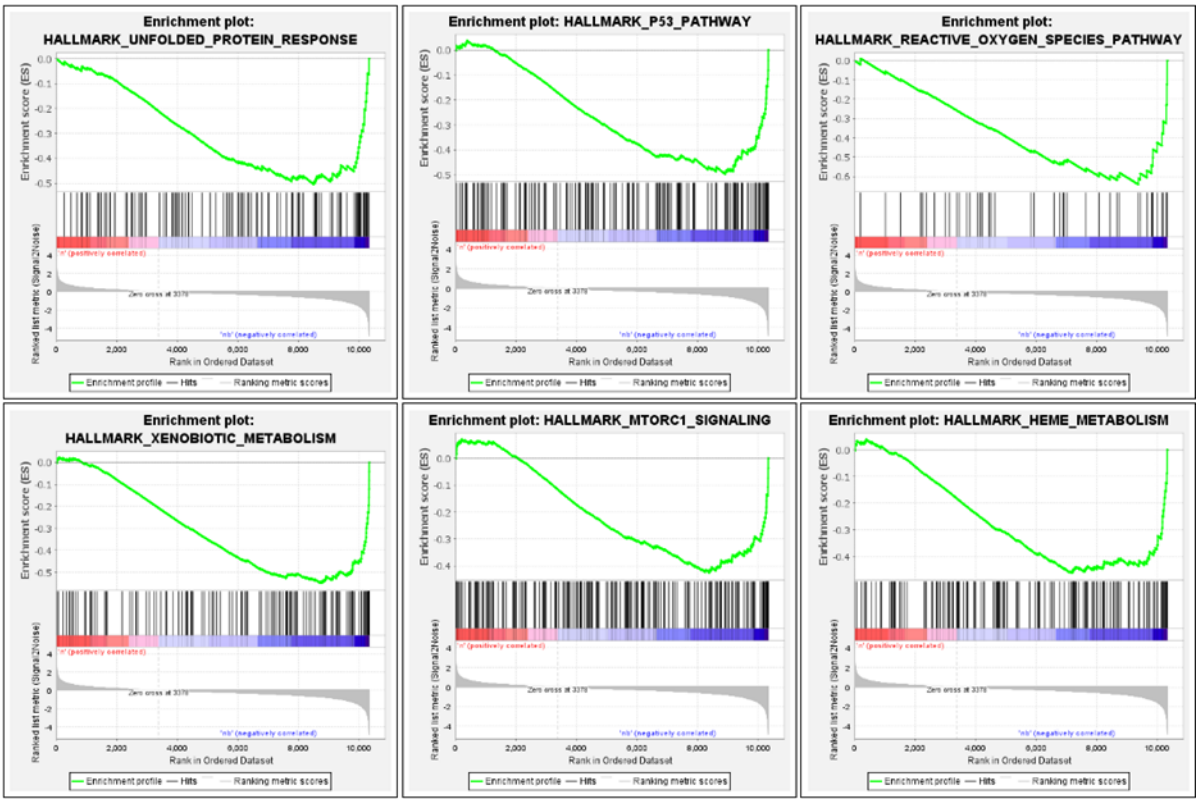


Figure 3.8. Gene set enrichment analysis of I3B treated RAW264.7 cells. Gene set enrichment analysis barcode plots indicate the position of the genes in each gene set with blue colors representing those with positive Pearson correlations upon I3B treatment. Enrichment plots for the six hallmark gene sets with the highest normalized enrichment score are shown

3.3.5. Anti-inflammatory effects of I3B are partially NRF2-dependent

Based on the significant upregulation of *Hmox1*, its prevalence in leading edge analysis across multiple gene sets and collections, and abundance of literature documenting in its involvement in NRF2-regulated pathways, we further investigated the contribution of NRF2 in I3B signaling[189,196,221-224]. BMM were cultured from WT and NRF2$^{-/-}$ mice and pre-incubated with 10 or 20 μM I3B prior to LPS stimulation. The data show that I3B pre-incubation attenuated LPS-stimulated *Tnf* gene expression by 3.06-fold (p = 0.06) in BMM from WT mice, while in BMM from NRF2$^{-/-}$ mice, a non-

significant increase in *Tnf* expression was observed (**Figure 3.9a**). I3B also up-regulated *Hmox1* 3.1-fold in WT cells, while a non-significant decrease was observed in $NRF2^{-/-}$ BMM. Notably, I3B pre-incubation followed by LPS stimulation resulted in a 23-fold increase in *Hmox1* expression in WT BMM, while only a 5.2-fold increase was observed with $NRF2^{-/-}$ BMM.

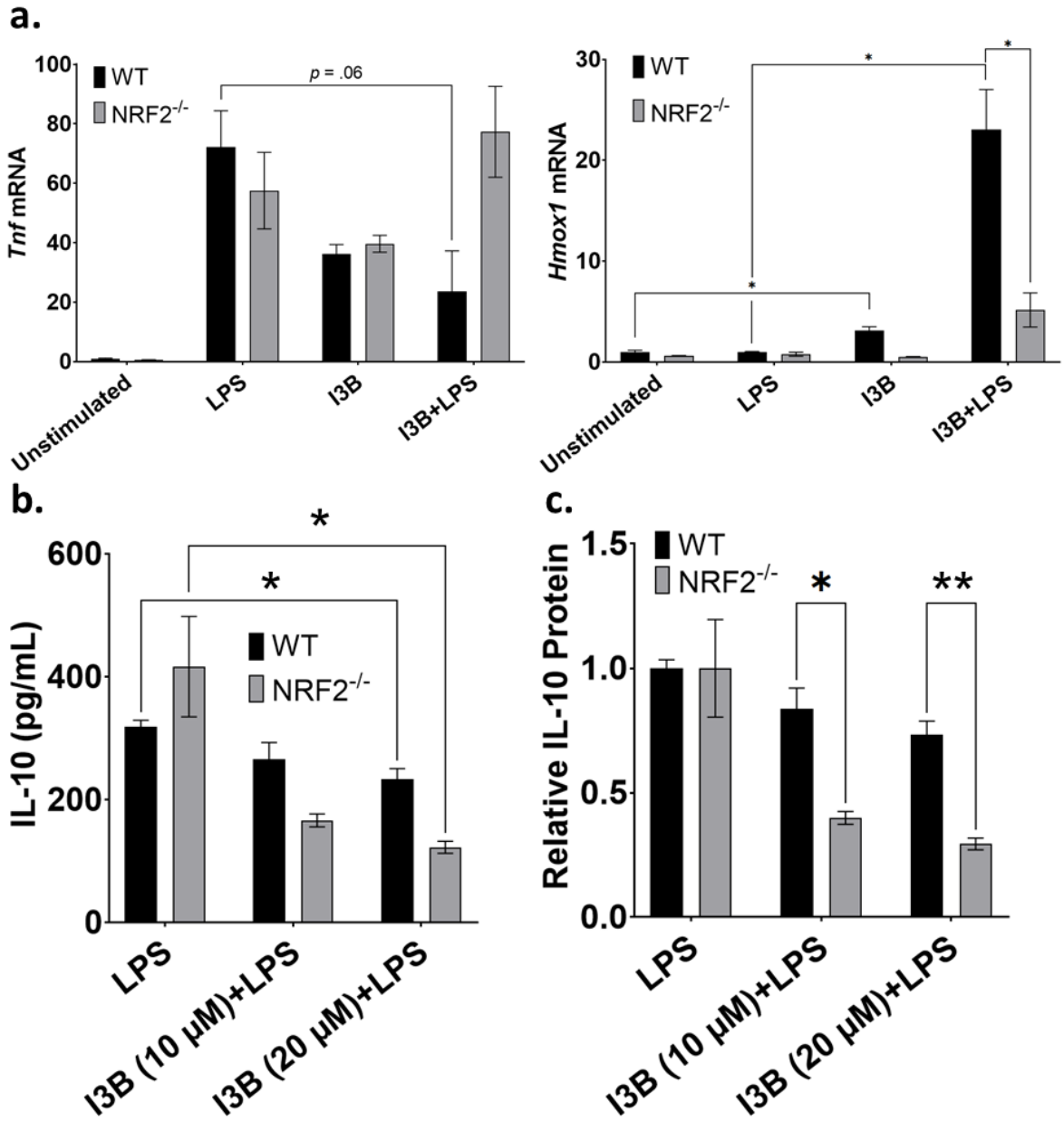
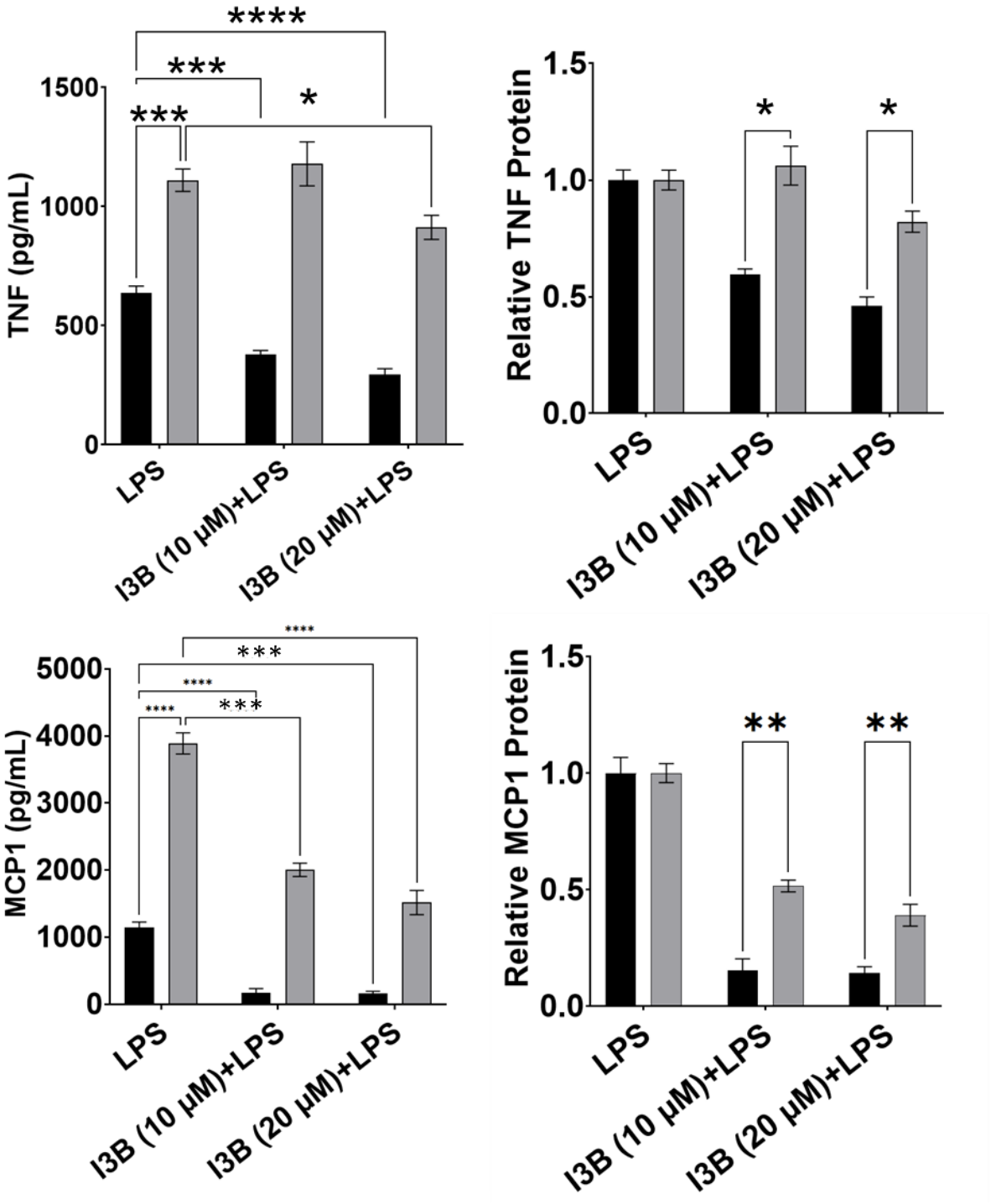


Figure 3.9. Anti-inflammatory effects of I3B in BMM are partially dependent on NRF2. **(a)** BMM from WT and NRF2$^{-/-}$ mice were pre-incubated with 20 μM I3B for 4 h followed by 1 h of 20 ng/mL LPS stimulation before mRNA isolation and qRT-PCR quantification. **(b, c)** BMM from WT and NRF2$^{-/-}$ were pre-incubated with 10 or 20 μM I3B then co-stimulated with LPS for 24 h before supernatant were isolated. Cytokines were quantified with a multiplexed antibody bead based assay. Absolute **(b)** and cytokine levels relative to the LPS control **(c)** are representative of three or four independent cell cultures. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$.



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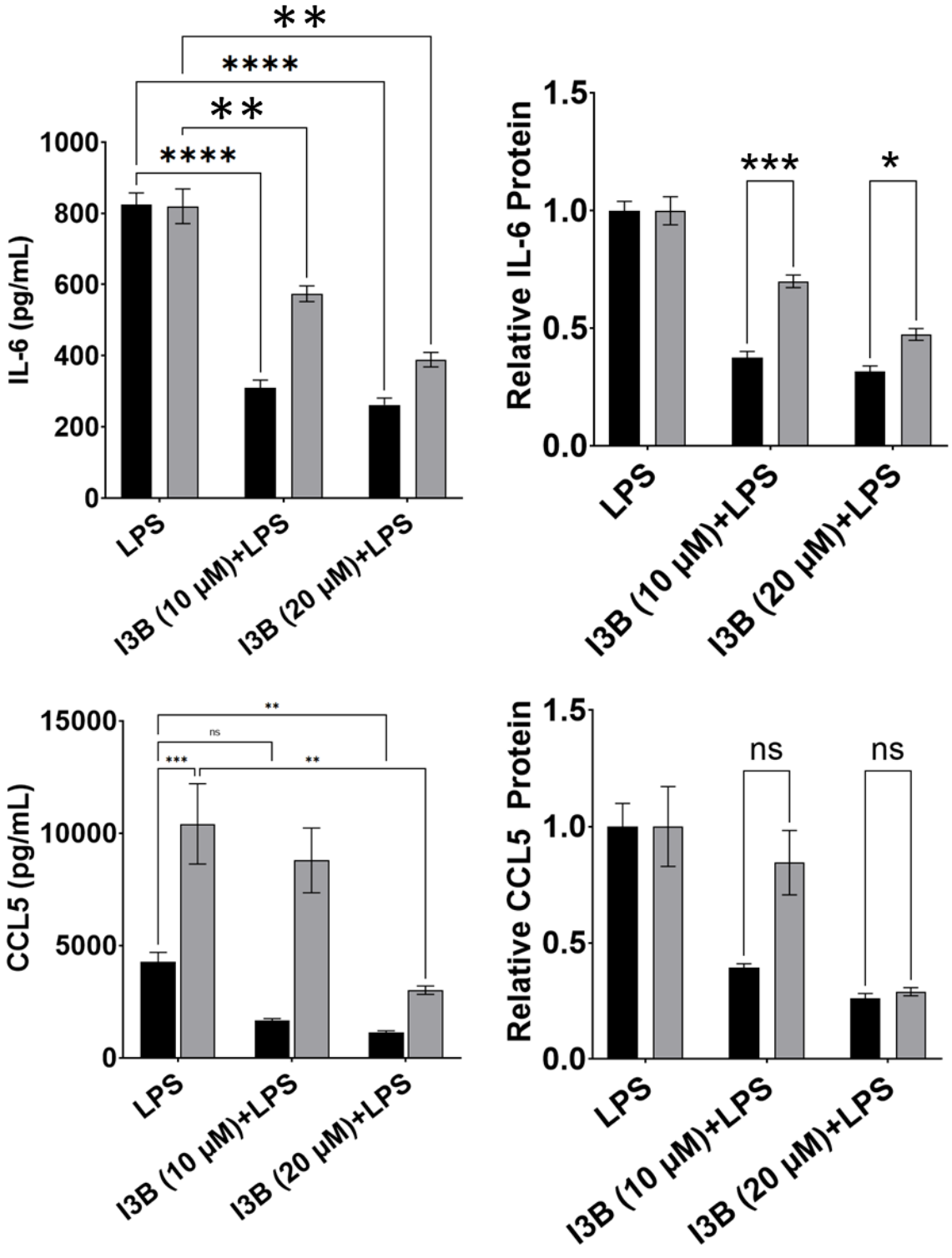

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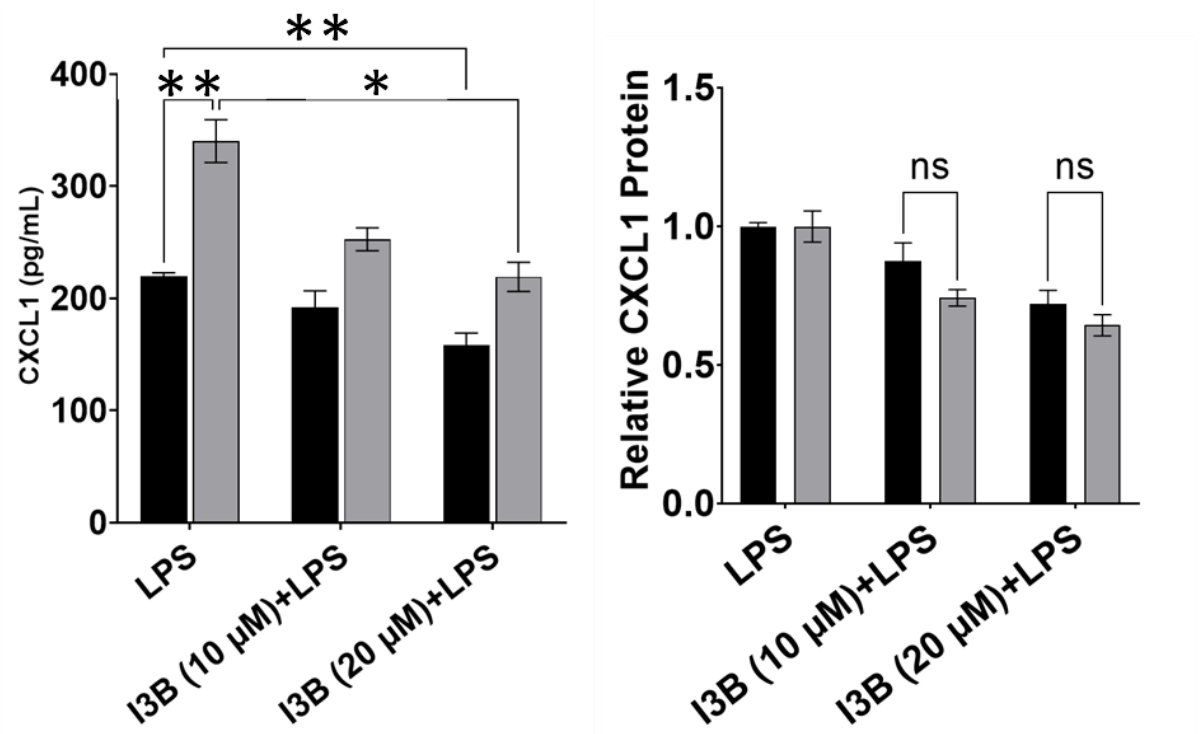

Figure 3.11. Continued.

The levels of secreted cytokines from WT and NRF2$^{-/-}$ BMM after I3B incubation for 4 h followed by 24 h LPS stimulation were analyzed using the LEGENDPlex^{TM} multiplex panel. The basal secretion of all the detected cytokines, except for IL-6, was higher in NRF2$^{-/-}$ BMM relative to WT, suggesting a hyper-responsive phenotype in BMM from NRF2$^{-/-}$ mice (**Figure 3.9b**). When normalized to the LPS control, the reduction in secreted TNF, MCP-1, and IL-6 with I3B pre-exposure was significantly greater in WT BMM than in NRF2$^{-/-}$ BMM (**Figure 3.9c**). In contrast, the expression of IL-10 in WT BMM pre-incubated with 20 μM I3B was higher than NRF2$^{-/-}$ BMM. Together, these results strongly suggest that the anti-inflammatory effects of I3B in macrophages are partially dependent on NRF2 and independent of AhR.

3.4. Discussion

The contribution of MDTMs to host immune function is being increasingly recognized, with evidence of several indole and indole-like compounds having pleiotropic effects on various immune cells such as epithelial and T cells. While these studies have established the role of MDTMs in the regulation of inflammation, the underlying mechanisms are poorly understood. In this study, we report on anti-inflammatory effects of indole in macrophages at physiologically relevant concentrations, and, using a cell-impermeant indole conjugate, show for the first time that these effects are primarily mediated through a cell surface receptor.

Prior studies investigating the mechanistic effects of MDTMs have focused on AhR-dependent responses. Indole acts as a weak AhR agonist and an antagonist of TCDD-stimulated AhR responses in epithelial cells and hepatocytes, and some of the protective effects of IAld on colonic inflammation have been shown to be AhR dependent[41,44,45,61,99,125,130,131,186,187]. However, our data showing that indole attenuates LPS-induced TNF in BMM from WT and $AhR^{-/-}$ mice and the inability of an AhR antagonist to affect indole-mediated reduction in TNF levels in RAW264.7 cells, and the lack of classical *Cyp1a1* induction by multiple AhR ligands in these cells suggests that indole's effects in these macrophages are AhR-independent. These observations underscore the importance of cell-context dependent signaling by different MDTMs.

To further probe AhR-independent effects of MDTMs, we synthesized a cell-membrane impermeant analog of indole through conjugation of IAld to BSA.

Interestingly, the conjugated indole (I3B) was approximately 50-fold more potent in attenuating LPS-mediated induction of TNF in RAW264.7 macrophages and partially depends on the transcription factor NRF2 in BMM. The attenuation of LPS-induced cytokine production by I3B in BMM extend beyond just TNF, but also to other classical cytokines, such as MCP-1 and IL-6. The relative reduction in secretion of these pro-inflammatory factors were more pronounced in WT than in $NRF2^{-/-}$ BMM. Furthermore, relative levels of anti-inflammatory IL-10 remained higher in WT supernatants than in $NRF2^{-/-}$, suggesting the anti-inflammatory potential of I3B is partially dependent on NRF2.

NRF2 is a transcription factor that regulates cellular responses to numerous stimuli, including oxidative and electrophilic stress, heme metabolism, detoxification of xenobiotic compounds, and inflammation[225,226]. Activation of NRF2 results in its liberation from its cytosolic suppressor, Kelch ECH associating protein 1, and allows for nuclear translocation and up-regulation of ARE-regulated genes. Transcriptome profiling of I3B conditioned RAW264.7 macrophages identified the upregulation of the ARE-regulated phase II enzyme genes, *Hmox1*, *Slc7a11*, *Nqo1*, and *Gclm*, which function to modify and eliminate numerous exogenous and endogenous compounds[227]. NRF2 can regulate inflammation indirectly through the elimination of ROS that can also induce inflammatory responses, but studies have has also demonstrated ROS-independent anti-inflammatory effects[198,224,226]. Direct binding of NRF2 to non-ARE sites in the proximity of some LPS-regulated cytokines in macrophages has been shown to inhibit their expression and RNA polymerase recruitment[198].

Of the ARE-regulated genes upregulated by I3B, *Hmox1* demonstrated the highest induction (18-fold). The primary enzymatic function of heme-oxygenase 1 (HO-1), the enzyme encoded by *Hmox1*, is to catalyze the conversion of free heme to biliverdin, releasing carbon monoxide and ionic iron as by-products[223]. In addition to detoxifying accumulating heme, HO-1 contributes to anti-inflammatory signaling via the release of endogenous carbon monoxide[228], possibly through an IL-10 related mechanism[222]. The potent induction of *Hmox1* led us to hypothesize that the anti-inflammatory effects of I3B may be mediated directly by HO-1 activity. However, experiments with a competitive inhibitor of HO-1, tin protoporphyrin IX, did not result in significant changes in I3B-mediated reduction of LPS-stimulated TNF accumulation (data not shown). A similar lack of effect was observed using an IL-10 neutralizing antibody (data not shown), suggesting that induction of HO-1 alone may not be sufficient to account for the anti-inflammatory effects observed with I3B.

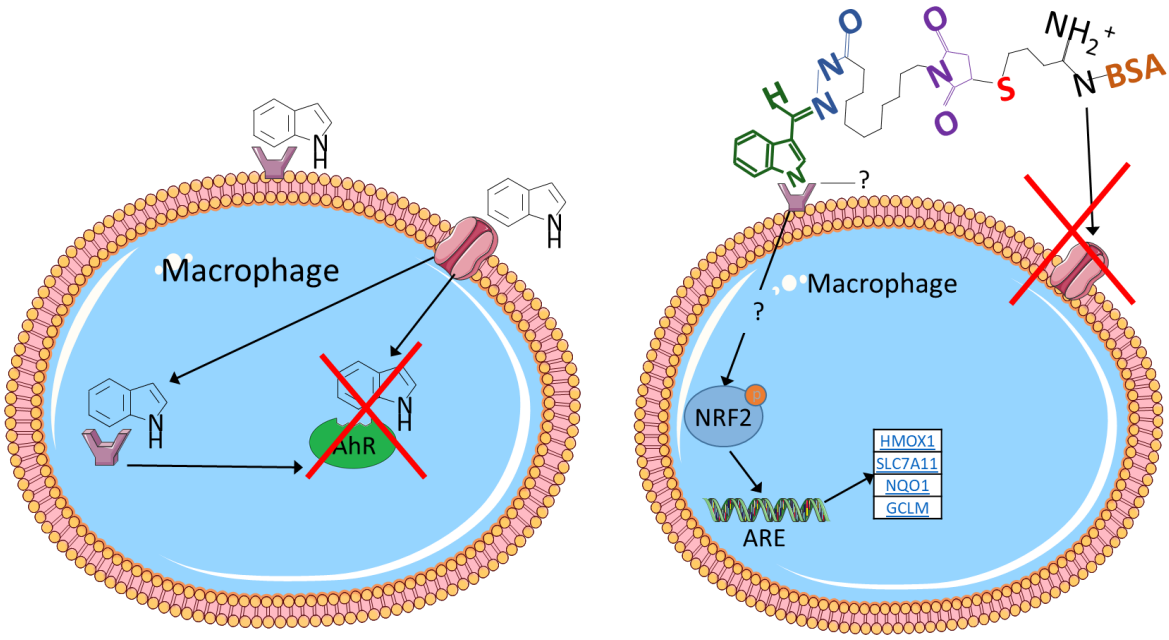

Figure 3.10. Schematic of proposed mechanisms involved in indole and I3B signaling in macrophages. Indole and I3B may engage the same surface receptor, however indole may be translocated to the cytosol and interact directly with cytosolic receptors as well. Intracellular uptake of I3B is sterically hindered, limiting its interaction to cell surface-mediated effects, resulting in NRF2-mediated signaling.

It is possible that the potent effects of I3B could be due to electrophilic activation of NRF2 resulting from residual KMUH in the I3B preparation. However, since a partially synthesized molecule consisting of BSA bound to KMUH, but lacking an indole moiety, demonstrated significantly reduced anti-inflammatory activity relative to I3B, it is unlikely that the observed effects are due to non-specific activation of NRF2. Since NRF2 is a transcription factor localized to the cytoplasm when inactive, and translocates to the nucleus upon phosphorylation, direct engagement of NRF2 with the cell membrane impermeant I3B is unlikely. This suggests the involvement of a novel surface-mediated signaling mechanism in mediating the anti-inflammatory effects of I3B

in macrophages. The observation that I3B inhibits LPS-stimulated TNF accumulation at concentrations 50-fold lower than free indole suggests a model where cell surface engagement of indole triggers more potent anti-inflammatory signaling than what free indole does through engagement with intracellular receptors (**Figure 3.10**). In this model, a balance exists for free indole between binding to cell-surface receptor(s) and intracellular uptake facilitated by diffusion or active transport, and this balance is likely shifted towards cell surface interactions when indole uptake is reduced with the I3B conjugate.

In summary, our results demonstrate anti-inflammatory properties of indole in macrophages that function independent of the AhR. We also report a novel cell-surface receptor mediated mechanism using the cell-impermeant I3B conjugate that depends in part on NRF2-mediated signaling. Identification of cell surface receptor(s) that are involved in mediating the effects of indole and other MDTMs could potentially lead to new strategies for using MDTMs or their synthetic derivatives as therapeutic molecules.

3.5. Materials and Methods

3.5.1. Animals and cell lines

RAW264.7 macrophages were purchased from ATCC (Manasassas, VA) and cultured in DMEM supplemented with glucose, penicillin, streptomycin, non-essential amino acids, HEPES, and 10% FBS at 37 °C and 5% CO_2 on 10 mM tissue-culture treated dishes. Cells were typsinized for passage at roughly 80% confluence and seeded

into tissue culture plates for experiments. Cells seeded in well-plates were cultured overnight before exposure to experimental conditions.

C57B/6 mice-derived wild-type and NRF2$^{-/-}$ mice femurs and tibias were provided by Dr. Venkatakrishna Rao Jala (University of Louisville). BMM were cultured as previously described [229]. Briefly, cells were flushed from the femurs and tibias of mice and cultured in 10 mM non-tissue culture treated dishes with RPMI containing 10 ng/mL macrophage colony-stimulating factor at 37 °C and 5% CO_2. Cells were fed additional media after three days of culture and harvested after seven days.

3.5.2. Flow cytometry

RAW264.7 cells were seeded at a density of ~2.7 x 10^4 to 3.2 x 10^4 cells per well in a round bottom TC-treated 96 well plate. Culture media was removed, and cells were washed with 200 µL cold PBS supplemented with 5% BSA twice and fixed with 200 µL 4% PFA for 20 minutes on ice. Cells were permeabilized with 200 µL BD Perm/Wash Buffer (BD Biosciences) for 20 minutes. Non-specific antigen binding was blocked with 0.1 µg Anti-Mouse CD16/CD32 (BD Biosciences, CA) in 75 uL for 5 minutes before cells were stained with TNF Rat anti-Mouse, Alexa Fluor 700, Clone: MP6-XT22 (BD Biosciences) for 30 minutes. Stained cells were resuspended in PBS and analyzed in a BD LSRFortessa™ X-20 and processed in FlowJo software (BD Biosciences).

3.5.3. Synthesis of I3B

Indole-3-aldehyde dissolved in dimethyl formamide (DMF) was reacted with KMUH in DMSO at a 5:1 molar ratio at room temperature on a shaker for 2 h. BSA was dissolved in PBS supplemented with 2 mM EDTA and the pH adjusted to 6.8. The concentration of this solution was used to calibrate BSA protein quantification using a Nanodrop 2000 by measuring absorbance at 280 nm wavelength. The KMUH-IAld reaction product was reacted with BSA at a 5:1 molar ratio (KMUH equivalents to BSA) on a shaker at room temperature for 2 h. Unreacted reagents were removed with a ZEBA desalting column (ThermoFisher) prepared according to manufacturer recommendations. The desalted product was concentrated in an Amicon® Ultra filter unit (3 kDa cutoff) prepared according to manufacturer recommendations at 4300 x *g* in a swinging-bucket rotor centrifuge for 1 h. The volume of the concentrated product quantified using a Nanodrop 2000. Buffer-exchange with serum-free DMEM was carried out in the Amicon® Ultra at 4300 x *g* column in a swinging-bucket rotor centrifuge for 1 h. The volume of the concentrated product was measured, and fetal bovine serum (FBS) added to final concentration of 10%. The I3B concentrate was then sterilized through a polyethersulfone 0.45 µM syringe filter. Desalted I3B without buffer exchange was used for MALDI mass spectrometry analysis. Samples were analyzed in an AXIMA MALDI-TOF Mass Spectrometer (Shimadzu) in reflectron mode at the Protein Chemistry Laboratory, Texas A&M University.

3.5.4. Multiplexed cytokine analysis

RAW264.7 cells were cultured as previously stated for ICS-FACS protocols. Supernatants were collected at the end of 24 h LPS stimulation and centrifuged at 4 °C, 400 RCF for 10 minutes. Supernatants were separated from any debris and stored at -80 °C before analysis. Samples were assayed with a LEGENDplex™ Mouse Inflammation Panel (13-plex) with the use of a filter plate according to manufacturer recommendations. Samples from the bead-based assay were then analyzed with a BD LSRFortessa™ X-20 and data was processed using BioLegend's LEGENDplex Data Analysis Software.

3.5.5. Quantitative reverse transcription PCR

Cultured RAW 264.7 cells were rinsed with cold PBS and frozen at -80 °C before RNA isolation with E.Z.N.A. Total RNA Kit I, (Omega Bio-tek). Cells were lysed according to manufacturer protocols and homogenized with Omega Homogenizer Columns. Total RNA was quantified in a Nanodrop 2000. RT-qPCR was performed using qScript One-Step SYBR Green reagents in a Lightcycler 96 System.

Primer pair sequences

Hmox1, FW	5’-GGTGATGGAGCGTCCACA-3’
Hmox1, RV	5’-AGGAAGCCATCACCAGCTTAA -3’
Tnf, FW	5’-TCTCATGCACCACCATCAAGGACT-3’
Tnf, RV	5’-TGACCACTCTCCCTTTGCAGAACT-3’
Cxcl10, FW	5’- CCAAGTGCTGCCGTCATTT-3’
Cxcl10, RV	5’- TTTCATCGTGGCAATGATCTCA-3’

3.5.6. RNA-sequencing

Total were extracted from RAW264.7 cells (n = 4) using E.Z.N.A.® Total RNA Kit I. RNA integrity were assessed using Agilent 2200 TapeStation. Samples were prepared using TrueSeqRNA sample preparation for paired-end 150 bp reads in an Illumina NextSeq 500 (Molecular Genomics Workspace, Texas A&M University, College Station, TX). Adapter sequences were removed with Trimmomatic, version 0.39 allowing no mismatches and removing leading and trailing sequences with a quality score below 9. Trimmed sequences were mapped to the Mouse genome assembly GRCm39 obtained from NCBI and indexed in STAR. Raw counts were normalized using the trimmed mean of M values and differential gene expression were analyzed using EdgeR. Gene set enrichment analysis and leading-edge analysis were performed using GSEA software from MSigDB[230,231].

4. MECHANISMS OF INDOLE SIGNALING IN MACROPHAGES AND EPITHELIAL CELLS

4.1. Overview

Microbially-derived tryptophan metabolites have been shown to have immunomodulatory effects in multiple cell types. The most investigated mechanistic pathway in this regard involves AhR-mediated signaling. However, AhR function and expression levels vary between cell types. Here, we characterize the effects of indole on highly AhR responsive colon epithelial cells and RAW264.7 macrophages that are unresponsive to classical AhR ligands. We find indole and a cell-impermeant indole-BSA conjugate inhibit TCDD-mediated *Cyp1a1* gene expression. In macrophages, we find indole inhibits LPS-induced TNF protein levels independent of *Tnf* mRNA expression, TNF protein degradation, the phosphatidylinositide-3'-OH kinase pathway, the Akt pathway, the p38 mitogen-activated protein kinase, and the c-JUN NH_2-terminal protein kinase pathway. In addition, we fail to find evidence that indole induces *Tnf*-inhibitory microRNAs, miR-124 and miR181b.

4.2. Introduction

Colonic epithelial cells play a critical role in maintaining a physical barrier between the host and the external environment. A delicate balance in permeability must be maintained to optimize nutrient absorption in the GI tract and impeding invasion of pathogens and even symbionts that may otherwise act as opportunistic pathogens. Previous work in colonic epithelial cell lines have demonstrated that indole can protect

against DSS-induced colitis, augment production of anti-inflammatory IL-10, downregulate TNF-induced expression of pro-inflammatory IL-8, and upregulating expression of genes involved in epithelial barrier function[39,40]. Studies have also linked AhR signaling to intestinal epithelial barrier function[232,233]. And indeed, some effects of MDTMs on colon epithelial cells have been found to be AhR dependent[219]. However, whether indole regulation of AhR signaling in epithelial cells is a result of direct AhR engagement has yet to be demonstrated. Here, we investigate both the agonistic and antagonistic effects of indole on a colon epithelial cell line, and in addition utilize a cell-impermeant indole-BSA conjugate, I3B, to limit direct AhR engagement.

In our previous section, we described our investigations into the signaling mechanisms of I3B in macrophages. Our findings identify NRF2 as a critical transcription factor in cell surface mediated effects of the compound, however free indole may act through both a surface receptor as well as through intracellular mechanisms upon cellular uptake. Here, we investigate other pathways of immune regulation by indole in stimulated macrophages. Phosphorylation events often mediate early responses to stimuli. Indeed, LPS stimulation in macrophages induces c-JUN NH_2-terminal protein kinase (Jnk), p38 mitogen-activated protein kinase (MAPK), and Akt kinase activity[234,235]. The activation of p38 MAPKs is critical for both initiation of inflammation and its subsequent resolution[236]. A conditional knockout of a p38 enzyme in murine macrophages resulted in reduced production of TNF and other cytokines upon LPS stimulation[237]. However, in immune resolution, anti-inflammatory IL-10 also induces p38 activity, leading induction of HO-1 and anti-inflammatory signaling [221].

Also involved in IL-10 mediated immune resolution are phosphatidylinositide-3'OH kinases (PI3Ks) and their cross talk with Akt kinases, with inhibition of these kinases leading to elevation of TNF levels[235,238,239]. PI3K activation also leads to down-regulation of LPS stimulated Jnk activation[235]. Whether indole may modulate these kinases in stimulated macrophages is unknown. Here, we utilize chemical inhibitors of these pathways to probe their involvement in indole-mediated anti-inflammatory signaling.

In our work, we find no changes in *Tnf* mRNA levels with indole pretreatment, despite a reduction in TNF protein levels. Another possible avenue of inflammatory regulation is through post-transcriptional degradation of cytokines. TNF protein can be broken down through both protease-mediated and lysosome-mediated pathways[240]. We utilize chemical inhibitors of both degradation systems to assess their involvement in indoles inhibition of TNF protein levels. Alternatively, miRNA regulation of *Tnf* translation may also explain our observations. MicroRNAs can destabilize the mRNA of their target genes but may also function through translational repression[205,206,209]. Indeed, miRNA-mediated TNF protein regulation independent of mRNA levels have been observed in macrophages[207,208]. We measure the induction of two miRNAs with potential to target *Tnf* by indole as a possible mechanism of its anti-inflammatory properties.

4.3. Results

4.3.1. Indole and I3B antagonizes AhR signaling in epithelial cells

To investigate the effects of indole and I3B on epithelial cells, young adult mouse colonic (YAMC) cells were pretreated with our compounds of interest and changes in *Cyp1a1* gene expression were measured. Indole inhibits TCDD-mediated *Cyp1a1* gene expression in a dose dependent manner, with 42% and 68% reductions at as 500 μM and 1 mM concentrations, respectively (**Figure 4.1a**). I3B exhibited potentiated antagonism at lower concentrations, with 64% and 86% reduction in *Cyp1a1* expression at 10 μM and 20 μM, respectively (**Figure 4.1b**). Previous work has identified low levels of *Cyp1a1* induction by indole in epithelial cell lines[41,61]. However, as shown in Section 3, upon BSA conjugation, agonistic effects were not observed.

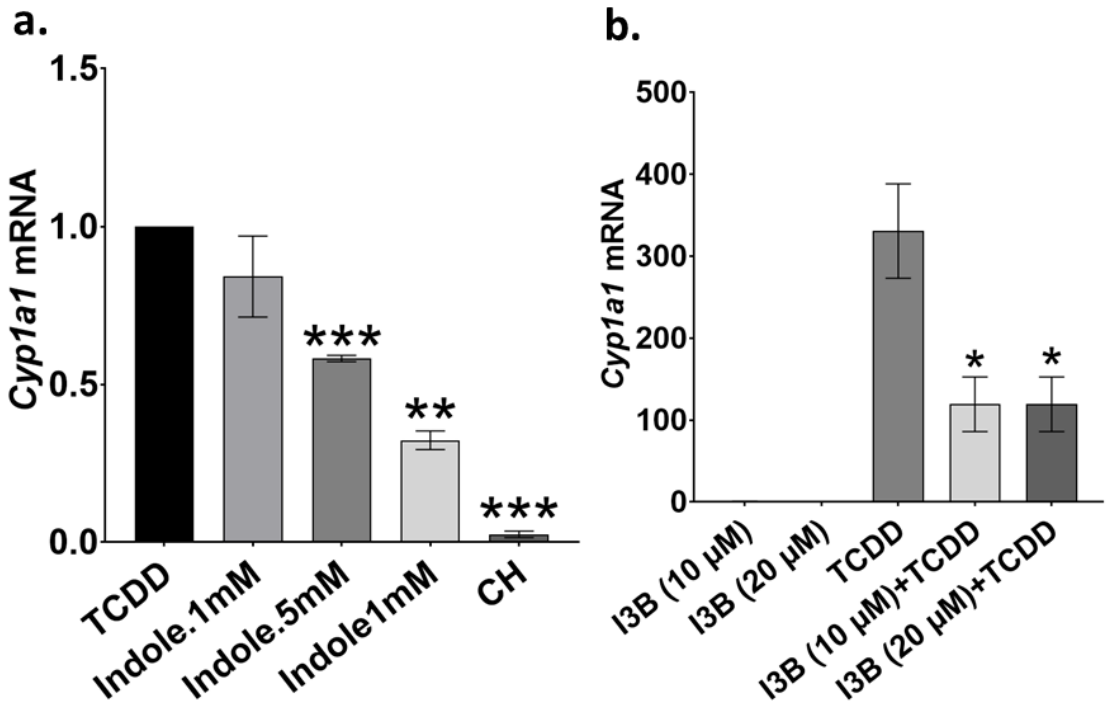

Figure 4.1. Indole and I3B antagonize TCDD-mediated Cyp1a1 expression. YAMC epithelial cells were pretreated with **(a)** indole or **(b)** I3B at varying concentrations for 4 h followed by co-stimulation with 10 nM TCDD for an additional 24 h unless indicated otherwise. Expression levels are normalized to the TCDD stimulated control in **(a)** and to the unstimulated control in **(b)**. CH: 20 μM CH-223191. ** $p < .01$, *** $p < .0001$.

4.3.2. Mechanism underlying anti-inflammatory effects of indole in macrophages

MAP kinases and Jnk family kinases are known to regulate pro-inflammatory gene expression in macrophages, and inhibition of Akt family kinases or PI3K have been shown to attenuate LPS induced inflammation[221,234-239]. We utilize chemical inhibitors of these pathways to investigate dependency of indole on kinase function. All the kinase inhibitors alone led to a reduction in LPS-induced TNF protein levels in RAW264.7 macrophages (**Figure 4.2a**). Normalizing to these levels, the additional reduction by pre-

incubation with indole, IAld, and I3B showed no significant reduction. In IAld primed cells, a slight potentiation of TNF reduction was observed with p38 MAPK inhibition.

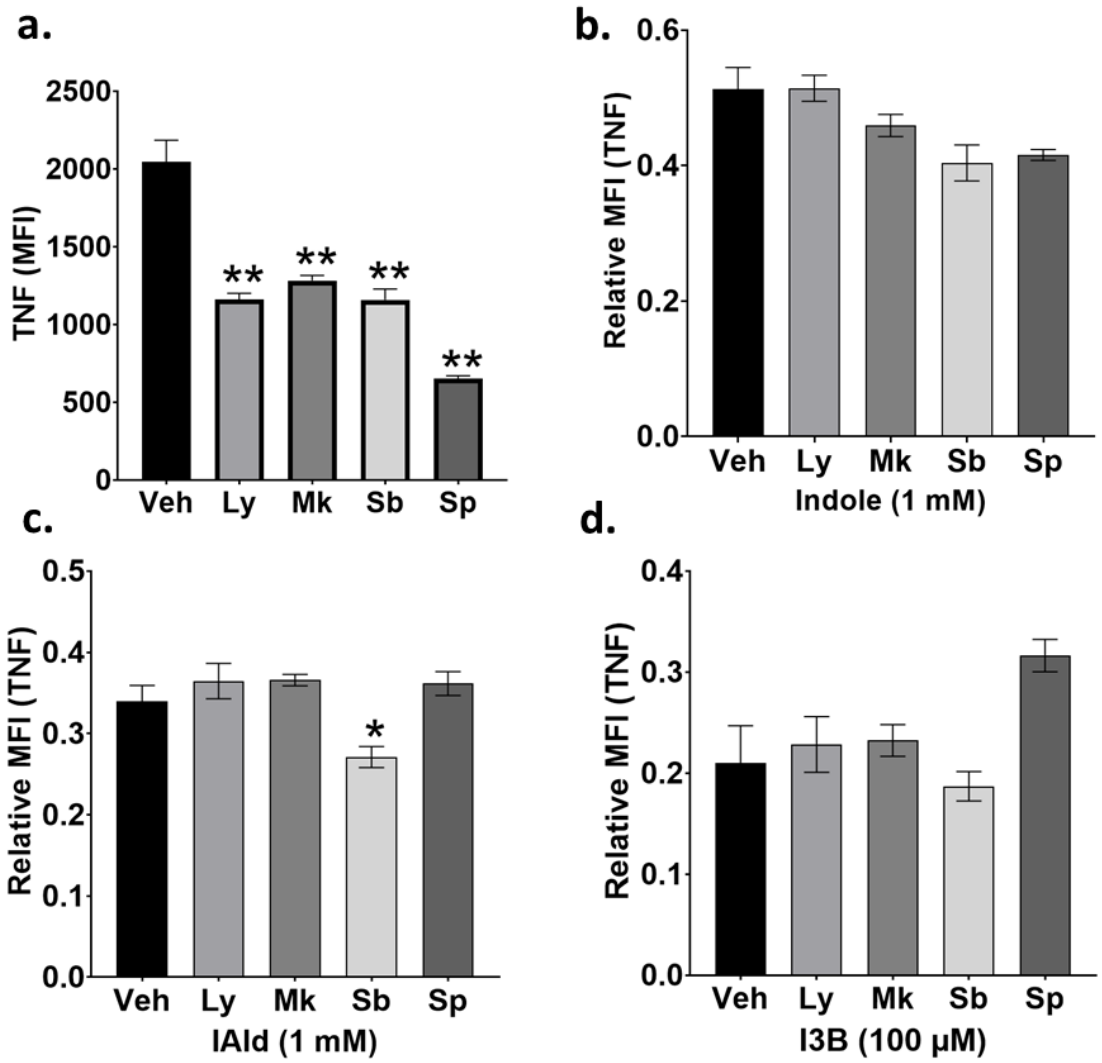

Figure 4.2. Effects of kinase inhibitors on MDTM-mediated reduction in TNF accumulation. RAW264.7 macrophages were pre-incubated with various kinase inhibitors for 1 h, followed by incubation with indole, IAld, I3B, or vehicle control for an additional 4 h. Lastly, cells were stimulated with 250 ng/mL LPS for the final 6 h of culture. **(a)** Changes in TNF accumulation caused by the kinase inhibitors alone were quantified using ICS flow cytometry. Reductions in TNF accumulation by **(b)** indole (1 mM), **(c)** IAld (1 mM), **(d)** I3B (100 μM) were normalized to the uninhibited control. Ly: PI3K inhibitor LY94002 (25 μM). Mk: AKT inhibitor MK-2206 (10 μM). Sb: p38 MAPK inhibitor SB202190 (10 μM). Sp: JNK inhibitor SP600125 (25 μM).

Time course analysis of LPS-stimulated *Tnf* gene expression reveal maximal induction at 1 or 1.5 h after stimulation, with roughly 14 and 13-fold induction, respectively (**Figure 4.3a**). Pre-incubation of cells with 1 mM indole had no statistically significant effect on subsequent LPS-stimulated *Tnf* expression for 1 or 1.5 h.

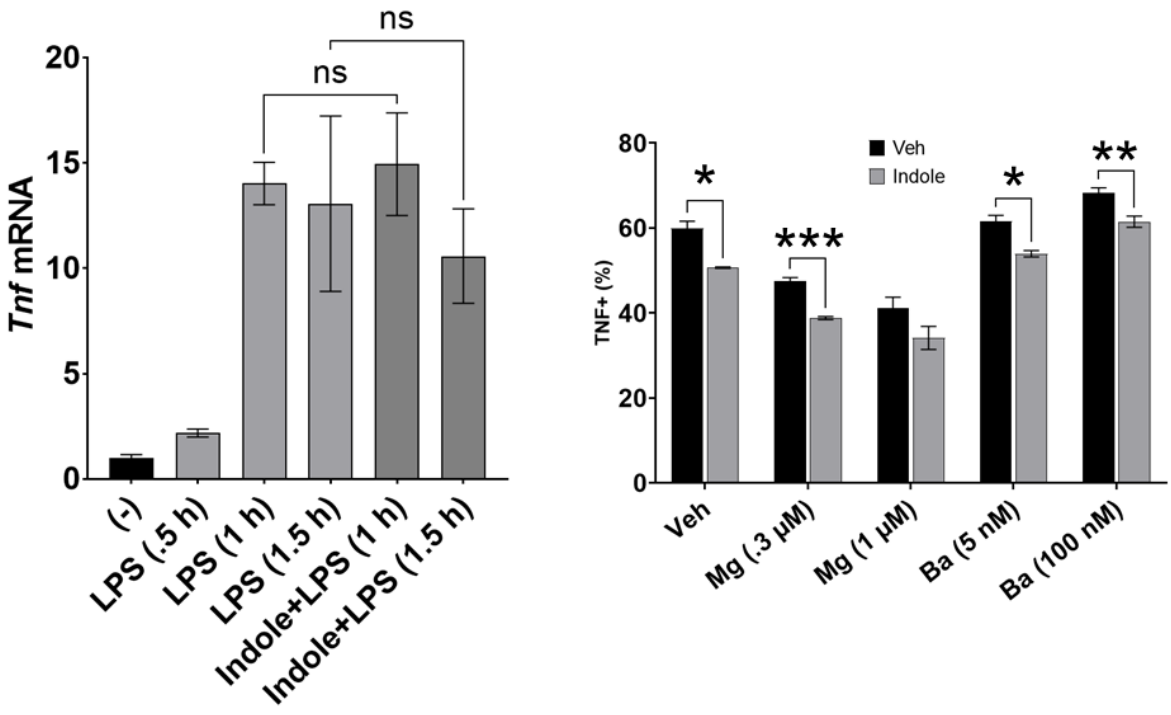

Figure 4.3. Indole regulation of TNF accumulation is independent of protein degradation. RAW264.7 macrophages were incubated with LPS **(a)** for varying durations with or without 1 mM indole for a total incubation time of 4.5 h. **(b)** Cells were pre-incubated proteasomal inhibitor, MG-132 (MG), or bafilomycin (Ba) for 14 h. Cells were then co-incubated with 1 mM indole or vehicle control for 4 additional hours, followed by co-stimulation with 250 ng/mL LPS for 6 h. * $p < .05$, ** $p < .01$, *** $p < .001$.

A possible mechanism of indole-mediated reduction in LPS stimulated TNF protein levels is through proteolytic degradation. Therefore, we inhibited proteasomal

and lysosomal degradation pathways with the chemical inhibitors, MG-132 and bafilomycin (**Figure 4.3b**). Inhibition with MG-132 alone reduced LPS-stimulated TNF accumulation in a dose dependent manner. However, pre-incubation with indole before LPS stimulation still resulted in roughly 40% reduction in the relative populations of TNF^+ cells. Similar results were found upon incubation with 5 nM and 100 nM bafilomycin, with roughly 19% and 16% relative reduction. None of the indole conditions were statistically different from relative levels of reduction in the absence of proteolytic inhibition.

Micro RNAs (miRs) are known to directly regulate translation of mRNA molecules, including those of inflammatory cytokines[209]. Unpublished work from a collaborator (Dr. Sanjukta Chakraborty, Texas A&M University) show that indole modulated miR-221 in lymphatic endothelial cells. Therefore, we utilized qPCR to investigate the modulation of miR-221, as well as known *Tnf* regulators, miR-124 and miR-181b, by indole in RAW264.7 macrophages (**Figure 4.4**). However, no significant induction the three miRNAs was observed under the conditions tested.

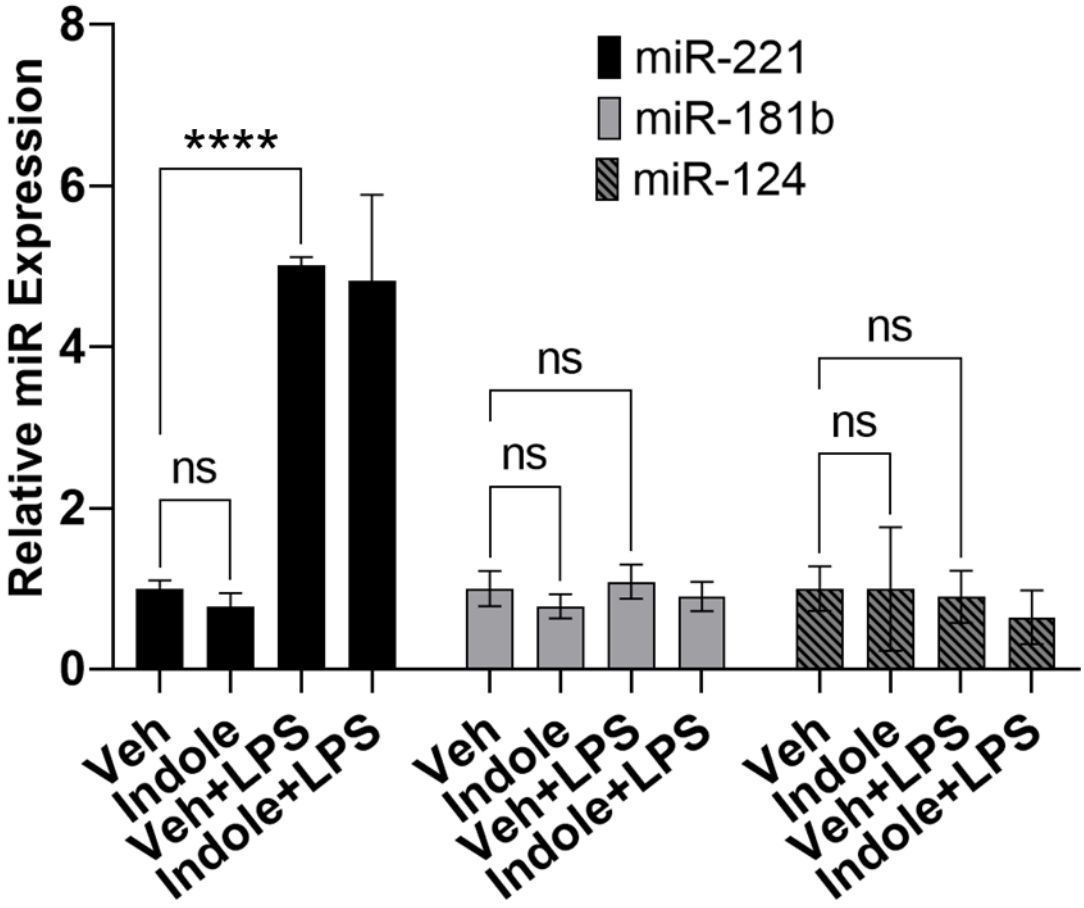

Figure 4.4. Effects of indole on microRNA gene expression. RAW264.7 macrophages were incubated with indole for 4 h, followed by 1 h of stimulation with 250 ng/mL LPS. Solid bars indicate unstimulated conditions, while slashed bars indicate LPS stimulation. Black indicates incubation with DMF control and green indicates incubation with 1 mM indole.

4.4. Discussion

Our findings that indole itself can also act as a potent inhibitor of TCDD-mediated *Cyp1a1* induction adds to previous observations that some MDTMs act as weak AhR agonists. Furthermore, conjugation of indole to BSA ablates the agonism seen with indole, in line with our hypothesis that BSA-conjugation significantly reduces

intracellular transport of indole, making direct engagement of the AhR unlikely. However, we observe a potentiation of AhR antagonism with I3B, akin to the potentiated anti-inflammatory effects we have previously discussed in macrophages. These results suggest an indirect mechanism of AhR regulation by indole stemming from a cell-surface receptor mediated mechanism in YAMC epithelial cells.

Our previous work identifies the involvement of NRF2 in the anti-inflammatory effects of I3B. Here, we sought to further investigate signaling mechanisms involved in the effects of unconjugated indole and IAld, which may involve a greater level of intracellular signaling. We probed the involvement of various kinases, namely PI3K, Akt family kinases, p38 MAPK, and Jnk family kinases, as these have been demonstrated to be involved in resolution of LPS induced inflammation[221,234-239]. Relative reductions in LPS stimulated TNF accumulation by indole, IAld, and I3B were not significantly altered by pretreatment with chemical inhibitors of these pathways, except for a mild potentiation of IAld by the p38 MAPK inhibitor, SB202190. These results demonstrate that the activity of these MDTMs and I3B are independent of these major kinase signaling pathways.

While we consistently observe reduction of LPS stimulated TNF protein levels with MDTM pretreatment, we did not observe a concomitant decrease in *Tnf* mRNA at any of the time points tested. A mechanism involving post-translational regulation would be consistent with these observations. To test the possibility that indole may upregulate degradation of TNF protein, we utilize inhibitors of two major proteolytic systems involved in TNF regulation, the proteasomal and lysosomal systems. Chemical

inhibition of either system did not negate the ability of indole to reduce TNF protein levels in RAW264.7 macrophages, suggesting degradation-independent mechanisms of indole regulation.

Another level of gene/protein regulation that may fit our pattern of observations is in that of miRNA-mediated inhibition of mRNA translation. We quantified gene expression a miRNA known to be modulated by indole in lymphatic endothelial cells (miR-221, unpublished) and two miRNAs known to regulate TNF protein without significantly altering mRNA expression, miR-124 and miR-181b[207,208]. However, modulation of expression of these miRNAs by indole in RAW264.7 macrophages were not observed.

These results highlight MDTM-mediated regulation of AhR signaling in YAMC colon epithelial cells, and in the case of I3B, through an indirect mechanism. In macrophages, we eliminate some major kinase signaling pathways, as well as proteolytic degradation pathways as candidates for indole-mediated reductions in TNF protein levels. While we were unable to identify specific miRNAs through which this may be mediated, our experiments are not exhaustive, and suggest a high-throughput screen for indole-regulated miRNAs may be warranted.

4.5. Materials and Methods

4.5.1. Cell lines

RAW264.7 macrophages were purchased from ATCC (Manasassas, VA) and cultured in DMEM supplemented with glucose, penicillin, streptomycin, non-essential

amino acids, HEPES, and 10% FBS at 37 °C and 5% CO_2 on 10 mM tissue-culture treated dishes. YAMC cells were a gift provided by Dr. Clinton Allred (Texas A&M University) and were cultured in RPMI supplemented with Insulin-Transferrin-Selenium-Sodium Pyruvate (Gibco), penicillin, streptomycin, 4% FBS, and recombinant mouse IFN-γ (Sigma-Aldrich) in non-experimental conditions. Cells were cultured under permissive conditions at 33 °C and 10% CO_2 and then at 37 °C and 5% CO_2 for experimental conditions. For culture of both cell lines, cells were typsinized for passage at roughly 80-90% confluence and seeded into tissue culture plates for experiments. Cells seeded in well-plates were cultured overnight before exposure to experimental conditions. In experiments involving TCDD, the compound was gifted to us by Dr. Stephen Safe (Texas A&M University).

4.5.2. Quantitative reverse transcription PCR

Cultured cells were rinsed with cold PBS and frozen at -80 °C before RNA isolation with E.Z.N.A. Total RNA Kit I, (Omega Bio-tek). Cells were lysed according to manufacturer protocols and homogenized with Omega Homogenizer Columns. Total RNA was quantified in a Nanodrop 2000. RT-qPCR was performed using qScript One-Step SYBR Green reagents in a Lightcycler 96 System.

Primer pair sequences

Cyp1a1, FW	5'-GAAGAAGTTAATCAAAGAGCACTACAGG-3'
Cyp1a1, RV	5'-TGTGTCAAACCCAGCTCCAAAG-3'
Tnf, FW	5'-TCTCATGCACCACCATCAAGGACT-3'
Tnf, RV	5'- TGACCACTCTCCCTTTGCAGAACT -3'

4.5.3. Flow cytometry

RAW264.7 cells were seeded at a density of 2.7 x 10^4 to 3.2 x 10^4 cells per well in a round bottom TC-treated 96 well plate. Culture media was removed, and cells were washed with 200 µL cold PBS supplemented with 5% BSA twice and fixed with 200 µL 4% PFA for 20 minutes on ice. Cells were permeabilized with 200 µL BD Perm/Wash Buffer (BD Biosciences) for 20 minutes. Non-specific antigen binding was blocked with 0.1 µg Anti-Mouse CD16/CD32 (BD Biosciences, CA) in 75 µL for 5 minutes before cells were stained with TNF Rat anti-Mouse, Alexa Fluor 700, Clone: MP6-XT22 (BD Biosciences) for 30 minutes. Stained cells were resuspended in PBS and analyzed in a BD LSRFortessa™ X-20 and processed in FlowJo software (BD Biosciences).

4.5.4. MicroRNA qPCR

MicroRNA samples were extracted from cells using a miRNeasy Mini Kit (Qiagen) per manufacturer instructions. RNA integrity were quantified using a TapeStation (Agilent) in the Molecular Genomics Workspace (Texas A&M University, College Station, TX). From these samples, cDNA was synthesized using a miScript II

RT Kit (Qiagen) with help from the lab of Dr. Sanjukta Chakraborty. For miRNA amplification, primers miScript primer assays (Qiagen) were used, with RNU6-2 used as a housekeeping gene.

5. TRYPTOPHAN METABOLITES AND T CELL DIFFERENTIATION

5.1. Overview

$CD4^+$ helper T cells differentiate into specialized subtypes to facilitate a wide range of immune responses. An imbalance in helper T cell subtype populations, particularly in the relative abundance between pro-inflammatory T Helper 17 (Th17) cells and immune-suppressive regulatory T cells (Tregs), have been associated with numerous autoimmune and inflammatory conditions including IBD, multiple sclerosis, and rheumatoid arthritis. Th17-mediated inflammatory responses have been implicated as pathogenic factors in these disorders, highlighting the importance of understanding what factors affect T cell fate and its subsequent effects on health. Here we show that MDTMs can have dichotomous effects on *ex vivo* T cell differentiation, with indole augmenting Treg differentiation, and 5-hydroxyindole (5-HI) augmenting Th17 differentiation. Interestingly, both compounds also exhibit reciprocal effects, with indole inhibiting Th17 differentiation and 5-HI inhibiting Treg differentiation. We carried out transcriptional profiling studies using microarrays to understand the molecular basis of MDTM's effect on T cell differentiation. Ingenuity Pathway Analysis (IPA) of the microarray data identified significant enrichment of IRF3 and IRF7 activation and prostaglandin E receptor 4 (PTGER4) inhibition signatures in indole-Tregs. These enrichments were driven, in part, by upregulation of multiple interferon induced protein with tetratricopeptide repeats (*Ifit*) genes and type I interferons. In 5-HI augmented Th17 cells, activation of the LPS signaling pathway, Tnf pathway, and phorbol myristate

acetate activated signaling are predicted to be most enriched, suggesting 5-HI may act as a pro-inflammatory signal with effects similar to pathogen-associated molecular patterns in Th17 signaling. These enrichments were driven in part by upregulation of pro-inflammatory cytokine genes such as *Il22* and epidermal growth factor receptor (*Egfr*). These results identify two MDTMs with reciprocal effects on T cell differentiation and identify novel likely network regulatory nodes that have not been previously associated with these MDTMs or T cell differentiation.

5.2. Introduction

$CD4^+$ helper T cells play a critical role in maintaining balance in immune signaling in part through the secretion of cytokines with pleiotropic functions[170,241,242]. The production of a large repertoire of cytokines by the helper T cell population is facilitated by differentiation of naïve $CD4^+$ T cells into distinct subtypes (primarily Th1, Th2, and Th17 cells) defined in part by their cytokine production[165].

IFN-γ production by Th1 cells and IL-4 production by Th2 cells are critical for driving classical and alternative macrophage activation, respectively[243-246]. IL-2, a major Th1 cytokine, is involved in maintaining $CD8^+$ T cell populations as well as contributing to $CD4^+$ T cell differentiation[247]. IL-17 and IL-22 secretion from Th17 cells elicit cytokine production by epithelial and endothelial cells[248-253]. Ultimately, coordinated differentiation and stimulation of cytokine release in helper T cells facilitate immunity to diverse classes of pathogenic organisms. However, aberrant cytokine production

contributes to autoimmune and allergic disease pathology, emphasizing the importance of anti-inflammatory regulation upon elimination of pathogenic threats[254-257].

The highly specialized $CD4^+$ $Foxp3^+$ Tregs function to suppress the proliferation of the aforementioned effector $CD4^+$ and $CD8^+$ T cells, downregulate inflammatory cytokine production by macrophages, and inhibit intestinal inflammation, facilitating immune tolerance and resolution of inflammatory responses[175]. Imbalance in the relative populations of Tregs and effector $CD4^+$ T cells, particularly the T helper 17 (Th17) subtype, are implicated in the pathogenesis of several autoimmune diseases. Elevated levels of Th17 relative to Tregs are observed in human clinical data and mouse models of rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, asthma, and IBD[258-262]. The direct conversion of Tregs to pro-inflammatory Th17 cells have been observed in arthritis and positively correlates with severity of disease, highlighting the importance of understanding the factors that drive differentiation of function of these two reciprocal helper T cell subtypes[258].

The differentiation of naïve T helper cells to Th17 cells is controlled by the transcription factor RORγt and is driven by interleukin 6 (IL-6), a major cytokine produced by inflammatory macrophages, and low levels of the cytokine TGF-β that is produced by macrophages, T cells, and numerous and other immune and nonimmune cells[171,263]. The presence of interleukin-23 (IL-23) promotes maintenance and expansion of differentiated Th17 cells and are produced primarily by DCs and macrophages. Induced Treg differentiation is controlled by the transcription factor, Foxp3, and is driven by the combination of interleukin-2 (IL-2) that is produced mainly by T cells, and

high concentrations of TGF-β[264]. The involvement of a common cytokine (TGF-β), albeit at different concentrations, in the differentiation of both Tregs and Th17 cells, and feedback from T cells in the production of IL-2 and TGF-β suggests the involvement of autocrine and feedback signaling that may contribute to the balancing of the Treg/Th17 ratio.

Outside of these cytokines, other molecules have been demonstrated to augment or inhibit the differentiation of T helper cells into Tregs or Th17 cells. A specialized population of gut homing dendritic cells induces the differentiation of naïve T cells into Tregs in a retinoic acid receptor-dependent manner[265]. *Ex vivo* studies revealed the direct contribution of the dietary vitamin A metabolite, retinoic acid, not only to Treg differentiation but also to the inhibition of Th17 populations and IL-17 production[266]. Specialized intestinal macrophages are another potential source of retinoic acid, expressing genes involved in its biosynthesis at comparatively higher levels than splenic macrophages. Co-culture of naïve T cells and intestinal macrophages in the presence of TGF-β induced higher levels of Treg differentiation, and was dependent on both retinoic acid and IL-10[267]. These results highlight the role of dietary metabolite on gut-localized antigen presenting cells in mediating Treg/Th17 differentiation and maintenance of gut immune homeostasis.

In addition to cytokines, AhR ligands have also been shown to modulate the differentiation of $CD4^+$ T cells into both Tregs and Th17 cells. Kynurenine, a major host derived metabolite of tryptophan and a potent activator of the AhR, augments TGF-β induced differentiation of naïve $CD4^+$ T cells to Tregs in a AhR dependent manner[268].

Similarly, high concentrations of the environmental toxin and persistent AhR ligand, TCDD, induces Treg differentiation, while the high affinity but rapidly degraded endogenous AhR ligand, 6-formylindolo[3,2-b]carbazole (FICZ), augments Th17 differentiation[269]. This apparent dichotomy has been attributed to the difference in the persistence of the ligand and hence, duration of AhR activation. Doses of TCDD and FICZ injections were optimized in mice *in vivo* based on liver *Cyp1a1* expression, such that repeated administration of FICZ would mimic that of a single TCDD administration. Under this modified protocol, FICZ administration reduced 17 and increased natural Treg populations to a similar degree as was observed with TCDD administration. Concomitantly, a 25-fold lower dose of TCDD administration increased IL-17 secretion and Th17 expansion, demonstrating opposing effects of high and low levels of AhR stimulation[269].

Specific members of the murine microbiota that can potentially alter T cell homeostasis have been identified. The segmented filamentous bacteria, *Candidatus savagella*, is capable of inducing Th17 differentiation in the intestinal lamina propria, providing increased pathogen resistance, but raising the potential for exacerbating autoimmune inflammation[177,270,271]. While *C. savagella* is not present in the human microbiota, *Bifidobacterium adolescentis* has been identified from human samples and exhibits similar Th17-inducing capabilities in mice. Indeed, the colonization of *B. adolenscentis* in specific pathogen free mice exacerbates experimental autoimmune arthritis[272].

One mechanism by which gut microbes modulate T cell differentiation and function is through the metabolites that they produce. SCFAs have been shown to increase the abundance and homeostatic function of colonic, with butyrate augmenting differentiation of naïve $CD4^+$ cells into Tregs and attenuated T-cell dependent experimental colitis in mice[105]. In a model of psoriasis, topical application of sodium butyrate upregulated Tregs and down regulated expression of IL-17 and inflammatory markers[273]. Patients with multiple sclerosis present with dysbiotic gut microbiomes, elevated Th17 populations, reduced Treg populations, and low levels of propionic acid in circulation and in fecal samples compared to healthy controls[108]. Exogenous propionic acid supplementation increased the relative proportions of Tregs to Th17 and potentiated their immune-suppressive capabilities[56,106]. While clinical interventions for IBD based on SCFA modulation have demonstrated mixed results, the positive outcomes from SCFA enemas and the promotion of SCFA production via prebiotics or probiotics warrant further investigation into therapeutic potential of SCFA modulation in Crohn's disease and ulcerative colitis[104,274-277]. Another major class of microbially-derived metabolites that impact T cell differentiation are secondary bile acids. The lithocholic acid derivatives, 3-oxoLCA and isoalloLCA, are found in humans and mice, but are completely absent in germ-free mice, suggesting their production is microbiota-dependent[278]. 3-oxoLCA inhibits Th17 differentiation by directly binding RORγt, while isoalloLCA augments Treg differentiation through an indirect mechanism[278].

Previous work has demonstrated the ability of the MDTM indole to augment Treg differentiation and inhibit Th17 differentiation[43]. However, its mechanisms of

action are not understood. In this work, we used microarrays to investigate the transcriptome of indole differentiated Tregs and 5-HI differentiated Th17 cells as a first step in identifying the underlying mechanism.

5.3. Results

5.3.1. MDTMs modulate differentiation of naïve T cells to Tregs and Th17 cell types

Single cell suspensions from the spleen and mesenteric lymph nodes of mice were stained for CD4 and CD25 and identify naïve helper T cell populations ($CD4^+$ $CD25^-$) were isolated. Sorted (>97% purity) populations of $CD4^+CD25^-$ cells were cultured on αCD3 and αCD28 coated plates to simulated antigen-induced activation and stimulate proliferation. Exogenous cytokines were added to cell culture media three days before analysis to skew differentiation towards either Treg or Th17, as described in Methods. Under Treg skew conditions, 20% of the cell population was $Foxp3^+$ after three days of culture (**Figure 5.1a**). Addition of indole (250, 500, and 1000 μ M) augmented differentiation of naïve T cells to Tregs in a dose-dependent manner, with Treg populations reaching 59% with the addition of 1000 μM indole for a 3-fold increase from baseline. Conversely, indole exhibited inhibitory effects under Th17 skew conditions, reducing Th17 populations by roughly 6-fold. 5-HI demonstrated reciprocal effects on differentiation, augmenting Th17 populations in a dose dependent manner, increasing Th17 by roughly 2 and 3-fold at 6.25 μM and 25 μM concentrations, respectively. Effects were again reciprocal to indole in Treg skew conditions, with 25

μM 5-HI inhibiting Treg differentiation by 5-fold. These results identify two MDTMs that modulate the differentiation of naïve T cells in opposing fashion under multiple skew conditions.

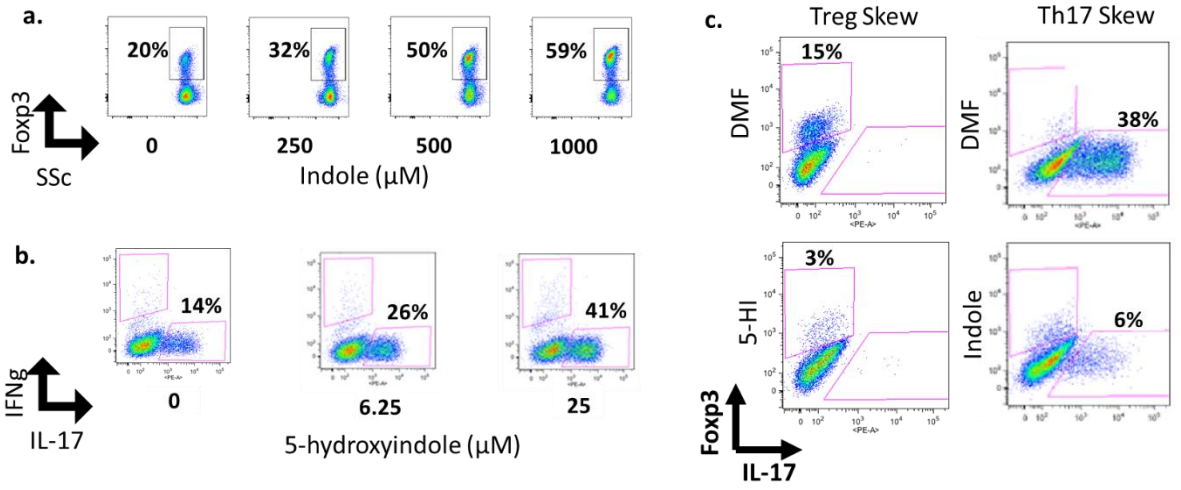

Figure 5.1. Microbially-derived tryptophan metabolites modulate T cell differentiation. $CD4^{+}CD25^{-}$ naïve T cells were sorted from the spleen and mesenteric lymph nodes of C57BL/6 mice and cultured on αCD3 and αCD28 coated 96 well plates. Cell culture media were supplemented with **(a)** 2 ng/mL TGF-β, 100 units/mL IL-2 for Treg differentiation or **(b)** 10 ng/mL IL-6, 5 ng/mL IL-23, 0.5 ng/mL TGF-β, 10μg/mL αIL-4, and 10 μg/mL αIFN-γ for Th17 differentiation and grown for three days. **(c)** Reciprocal effects of indole and 5-HI were tested at 1 mM and 25 μM, respectively. Intracellular staining for Foxp3and IL-17 were used to determine the relative populations of Tregs and Th17 cells, respectively.

We used microarrays to elucidate the transcriptional basis underlying the effect of indole and 5-HI on T cell differentiation. Specifically, we compared the differences in transcriptional signatures between Tregs and Th17s differentiated in the presence or absence of MDTMs. The *ex vivo* T cell differentiation experiment was repeated using *Foxp3*-GFP and *Il17a*-GFP transgenic mice and pure populations of conventional Tregs and indole-Tregs were sorted by identifying GFP^{+} populations. Principal component

analysis of the microarray data showed that both indole-differentiated Tregs and 5-HI-differentiated Th17 cells were significantly different from Tregs or Th17 cells differentiated without indole or 5-HI, respectively (**Figure 5.2a**). Nearly 90% of the variance in the data was captured by the first two principal components.

We further identified that 551 genes were differentially regulated in indole Tregs (>2-fold, *p-value* <0.05) and 219 genes in 5-HI differentiated Th17 cells. Amongst the most differentially regulated genes in indole-Tregs were *Il13* (82-fold down regulation) and *Tnfrsf8* (55-fold down regulation), and *Cxcl9* (45-fold upregulation) (**table 5.1**). In 5-HI differentiated Th17 cells, *Il22* was upregulated 16-fold, *Iltifb* 14-fold, and *Il9* was downregulated 12-fold (**table 5.2**). In both cases, cytokines appear be amongst the most differentially regulated genes. Heat maps of differentially regulated cytokines in indole-Tregs, reveal a large cluster of interleukins that are downregulated by indole, i.e. *Il10, Il5, Il24, and Il4* (35, 26, 14, and 10-fold, respectively), whereas multiple type I interferons are upregulated (**Figure 5.3a**). In 5-HI augmented Th17 cells, a cluster of genes encoding IL-3, IL-22, and IL-22b are upregulated, and generally, a greater proportion of cytokines were upregulated as compared to indole-Tregs (**Figure 5.3b**).

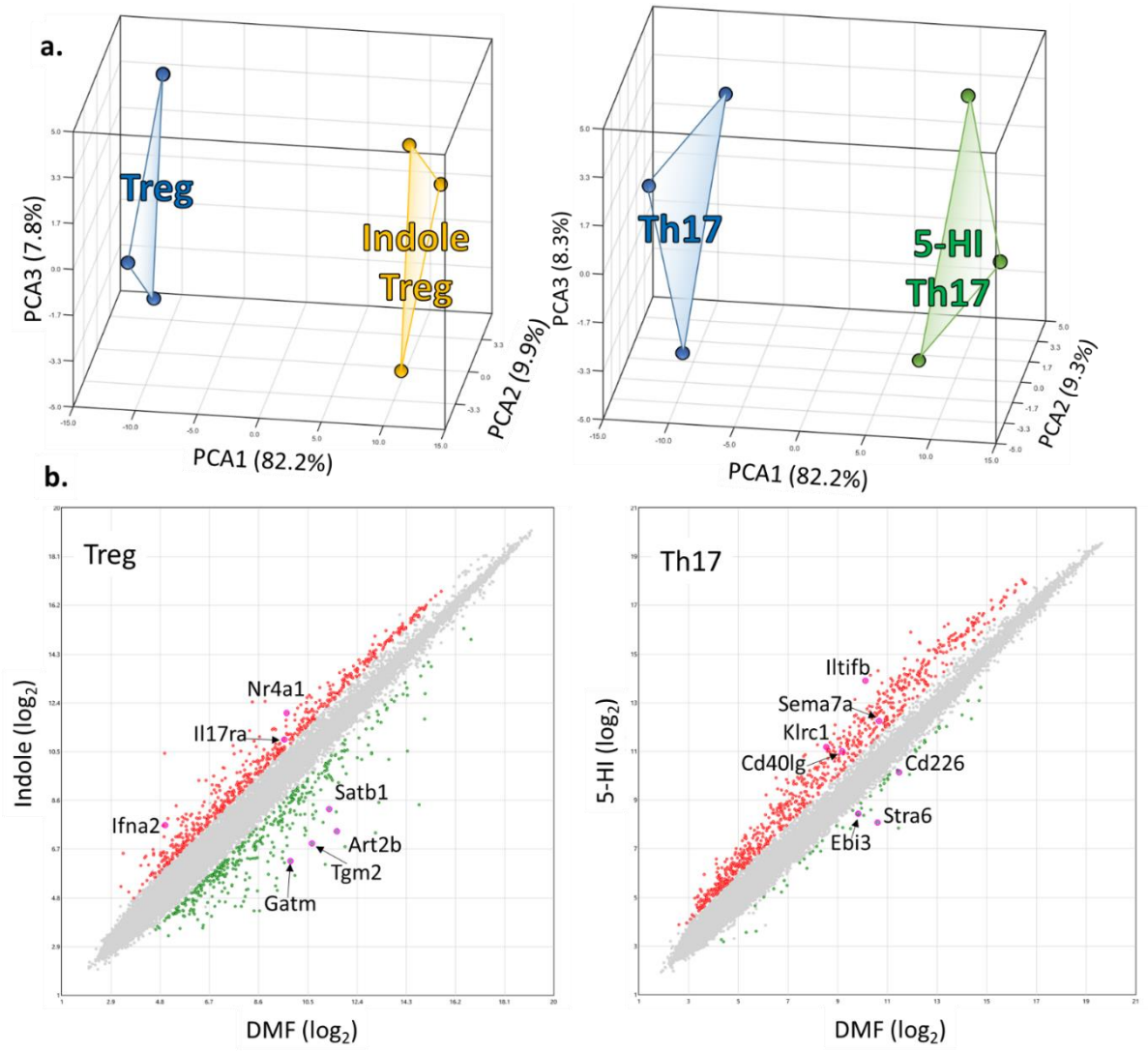

Figure 5.2. Transcriptome profiling of MDTM-differentiated T cells. (a) A three factor principal component analysis, which each data point representing RNA from a single mouse for a given condition. **(b)** Scatter plots of differentially regulated genes. Targets upregulated by indole (top) are shown in red, while down regulation is marked in green.

To identify potential upstream regulators that likely contribute to the observed transcriptional signatures, the differentially regulated genes were further analyzed using Ingenuity Pathway Analysis (IPA) using previously curated datasets available through

the program. Network nodes were scored based on their enrichment in the data sets and the nodes with the highest enrichment scores were further analyzed. The nodes with the highest absolute enrichment scores are listed in indole-Tregs, IRF3, a transcription factor known to regulate responses to type I interferons, was the most enriched node (**Figure 5.4a, Table 5.3)**. Of the 43 genes in the IRF3 signaling pathway, 35 genes were present in the indole Treg data set and showed a pattern of regulation that suggested increased IRF3 activation in indole-Tregs, resulting in a z-score of 5.0. The second most enriched network regulator is IRF7 with a z-score of 4.5. These two interferon regulatory factors are known to regulate the same type I interferons and have 32 overlapping genes used to generate activation prediction. The genes that supported activation of these two transcription factors were primarily upregulated cytokines, such as *Cxcl9*, *Ifit1*, *Cxcl10*, *Ifit3*, *Ifna4*, and *Usp18* (44.5, 8.44, 6.97, 4.48, and 4.22-fold upregulation, respectively). While the involvement of IRF transcription factors in promoting Treg differentiation is a novel finding, network analysis also predicts NR4A3 activation (z-score 2.4), a transcription factor known to be critical in thymic Treg differentiation[279].

Table 5.1 Top differentially regulated genes in indole augmented Treg cells

Gene Symbol	Fold Change	p-value	Gene Symbol	Fold Change	p-value
Cxcl9	44.5	6.30E-07	Il13	-82.31	4.76E-06
Nr4a3	16.94	7.00E-04	Tnfrsf8	-54.93	1.54E-06
Mir669b	8.99	8.37E-07	Il10	-35.05	9.09E-05
Ccrl2	8.74	2.20E-05	Gzma	-33.79	5.00E-04
Eomes	8.55	2.00E-04	Il5	-26.44	5.93E-05
Ifit1	8.44	3.85E-06	Pth	-20.22	4.00E-04
Pld4	7.84	1.37E-06	Cysltr1	-20.11	1.70E-07
Cxcl10	6.97	1.97E-05	Art2b	-18.6	1.26E-07
Gm14944	6.96	8.27E-07	Gm19585	-15.93	1.93E-06
Ifit3b	6.57	8.00E-04	Il24	-14.06	1.45E-05
Gramd3	6.37	1.45E-06	Tgm2	-13.45	3.66E-08
Ifna2	6.27	8.92E-08	Enam	-13.27	2.00E-04

Table 5.2 Most differentially regulated genes in 5-HI augmented Th17 cells

Gene Symbol	Fold Change	p-value
Il22	15.64	1.02E-08
Gm25930	13.94	1.64E-06
Iltifb	13.87	4.04E-08
Rnu73b	12.89	6.00E-04
Gm9847	10.85	3.00E-04
Gm22127	10.64	7.40E-05
Dnph1	9.12	3.00E-04
Gm23947	8.99	5.19E-05
Gm12801	8.69	4.00E-04
Lef1	8.62	4.00E-04
Gm24508	8.47	2.96E-07
Gm26430	8.13	9.50E-06

Gene Symbol	Fold Change	p-value
Il9	-12.05	5.18E-07
Stra6	-5.82	3.54E-07
Sult2a2	-4.7	2.27E-01
Ddx24	-4.34	9.20E-03
Mir6974	-4.12	7.72E-05
Gatm	-3.55	4.62E-05
Gm5648	-3.37	4.00E-04
Acat2	-3.35	4.63E-06
Usp18	-3.19	9.71E-05
Lta	-3.06	1.03E-05
Ighm	-2.85	4.28E-06
Ggnbp2	-2.84	4.20E-01

In 5-HI Th17 cells, amongst the nodes with the greatest z-scores and lowest *p-values* are LPS and TNF, with activation z-scores of 4.1 and 4.5, respectively (**Figure 5.4b, table 5.4**). The most differentially regulated genes contributing predicted activation of these pathways were *Il22, Egfr* (epidermal growth factor receptor) and *Lss* (Lanosterol synthase), with 15.6, 7.2 and 7.0-fold upregulation, respectively. These results may suggest 5-HI acts as a pro-inflammatory signal in its involvement in regulating Th17 differentiation.

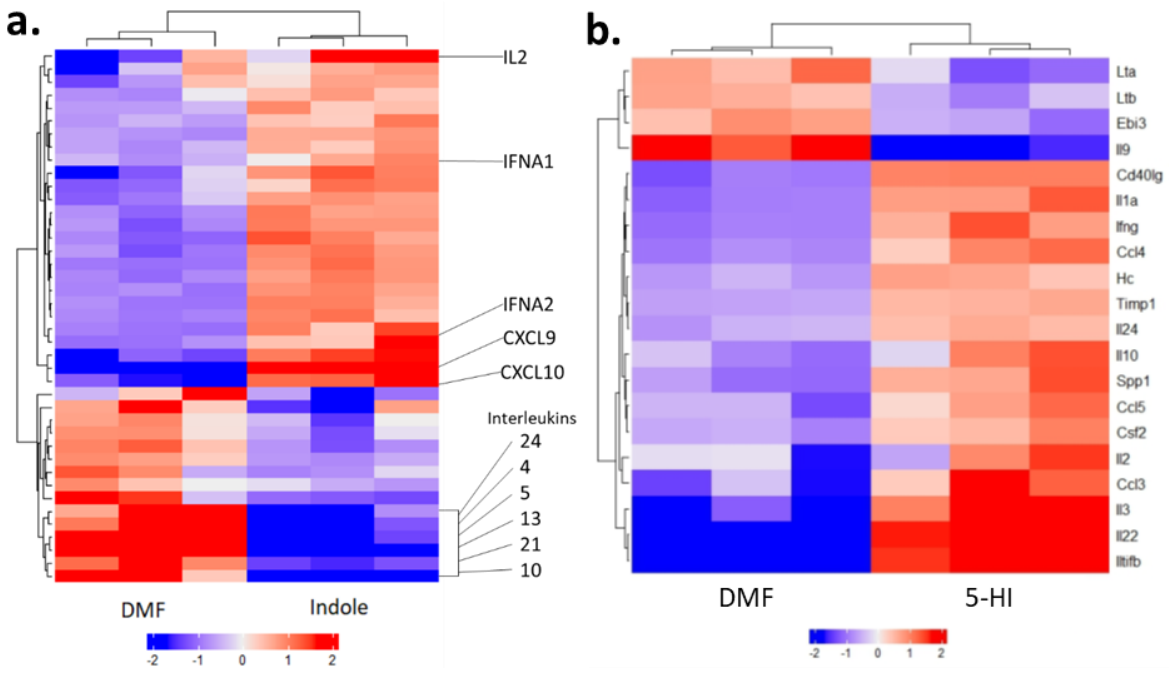

Figure 5.3. Heat maps of differentially regulated cytokines. Differentially regulated cytokines (fold change > 2, $p < 0.5$) by **(a)** indole in Tregs and **(b)** 5-HI in Th17 cells are clustered with row-wise normalization and color saturation at 1.6.

To our knowledge, direct involvement of IRF3 and IRF7 in mediating Treg differentiation has not been previously reported. As the top network regulators in 5-HI augmented Th17 cells seem to indicate a general up regulation of classical pro-inflammatory signaling, we focus on our findings in indole-Tregs as they may have higher potential for identifying novel signaling pathways. The trends observed in the microarray data set for changes in IRF3 and IRF7 signaling in indole Tregs were validated using qRT-PCR. While the microarray and qRT-PCR data did not have complete concordance, similar expression patterns were observed for several critical IRF-regulated genes. Specifically, indole upregulated *Ifna*, *Ifnb1*, *Ikbke*, and *Il13* similar

to the microarray data (**Figure 5.4c)**. These results together suggest MDTMs may play a unique role in T cell differentiation.

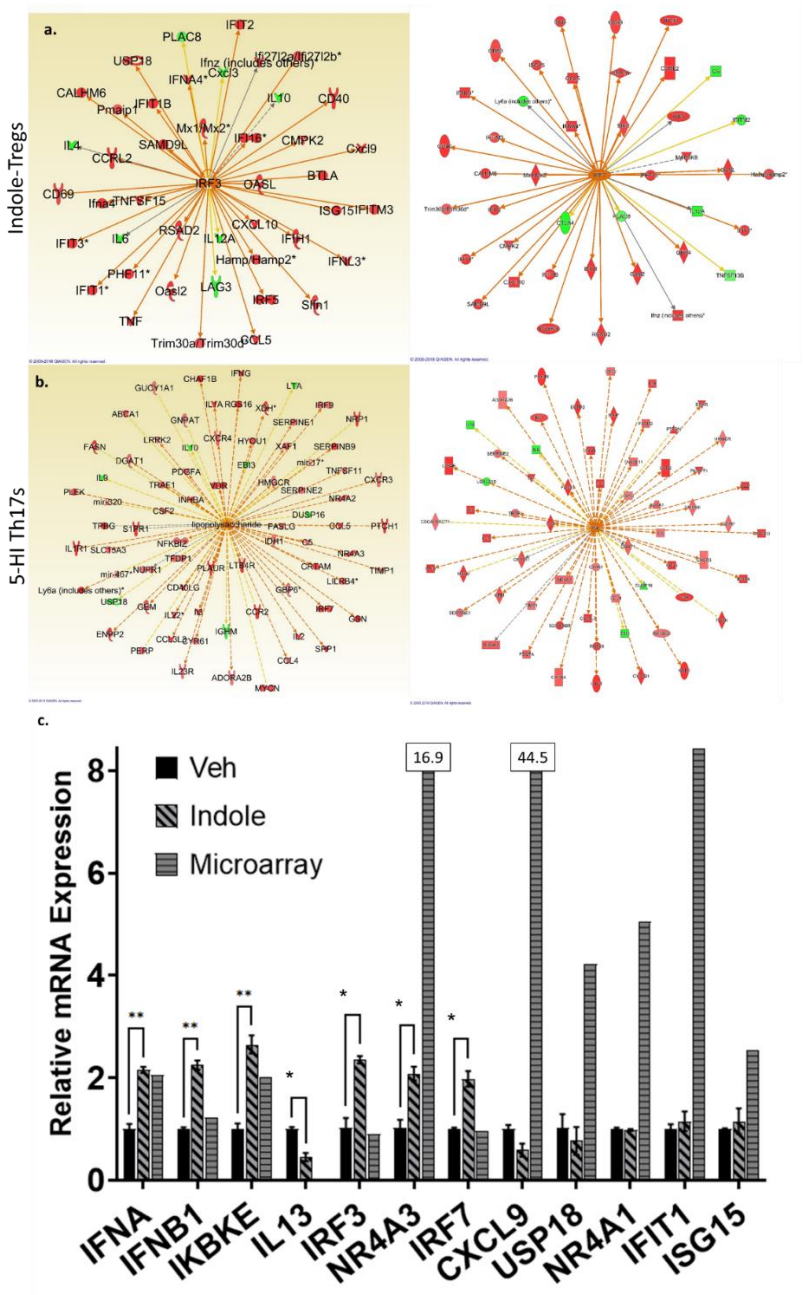

Figure 5.4. Network analysis of MDTM-differentiated T cell signatures. Ingenuity pathway analysis was used to integrate differential gene expression data to infer regulation of curated signaling nodes**. (a)** Network map of indole-Tregs with IRF3 and IRF7. **(b)** Network map of 5-HI Th17s with LPS and TNF. Red markers on genes indicate upregulation, while green markers indicate down regulation. Orange arrows indicate a direction of signaling consistent with activation, while yellow arrows indicate inconsistent patterns. **(c)** Comparison of relative gene expression in select genes via qRT-PCR or microarray analysis.

Table 5.3 Top upstream regulators of indole differentiated Tregs

Upstream Regulator	*p-value*	z-score	Molecule Type
IRF3	4.965	7.13E-32	transcription regulator
IRF7	4.528	7.83E-33	transcription regulator
PTGER4	-4.392	1.74E-19	g-protein coupled receptor
dexamethasone	-4.098	6.28E-20	chemical drug
ACKR2	-3.742	1.11E-13	g-protein coupled receptor
salmonella Minnesota R595 lipopolysaccharides	3.723	1.51E-19	chemical-endogenous non-mammalian
Interferon alpha	3.658	4.12E-30	group
ribavirin	3.628	8.75E-15	chemical drug
TLR3	3.587	1.51E-33	transmembrane receptor
STAT1	3.558	1.54E-32	transcription regulator
TICAM1	3.526	6.88E-24	other
Ifnar	3.514	7.27E-16	group

Table 5.4 Top upstream regulators of 5-HI differentiated Th17 cells

Upstream Regulator	*p-value*	z-score	Molecule Type
TNF	4.45	1.45E-12	cytokine
phorbol myristate acetate	4.126	4.36E-12	chemical drug
PD98059	-4.103	7.48E-08	chemical - kinase inhibitor
lipopolysaccharide	4.061	4.01E-21	chemical drug
SB203580	-3.801	2.07E-08	chemical - kinase inhibitor
Jnk	3.558	9.38E-07	group
EGF	3.556	8.37E-06	growth factor
bleomycin	3.544	3.02E-07	chemical drug
JUN	3.493	1.44E-06	transcription regulator
TREM1	3.486	2.24E-09	transmembrane receptor
SMARCA4	3.484	3.14E-06	transcription regulator
D-glucose	3.476	5.41E-02	chemical - endogenous mammalian

5.4. Discussion

The balance between Treg and Th17 cell populations is important in the context of inflammation in the GI tract. *Ex vivo* methods to differentiate naïve T cells to Tregs or Th17 cells with different cytokines are well established; however, the use of microbiota metabolites for T cell differentiation is not common[165]. Of the microbiota metabolites, SCFAs such as butyrate have been shown to promote the differentiation of naïve T cells to Tregs[105,106,273]. Conversely, the SCFA propionate has been found to inhibit Th17 populations *in vivo*[107,108]. In this work, we identify MDTMs as a class of molecules that can have diverse effects on T cell differentiation. Our data show that indole not only augments the development of Tregs but also inhibits the differentiation of Th17 cells. This observation suggests that indole promotes immune tolerance while 5-HI elicits the reciprocal response, inhibiting Treg differentiation and augmenting Th17 differentiation, and thus, promoting a pro-inflammatory state.

Transcriptome profiling and IPA suggested that the transcription factor IRF3 is involved in mediating indole signaling. Expression of a phosphomimetic form of IRF3 in dendritic cells has previously been shown to induce IL-10 secretion, and indirectly promote expansion of Tregs *in vivo*[280]. Contrary to those results and our own, viral induction of IRF3 was found to suppress Treg differentiation[281]. However, to our knowledge, direct involvement of IRF3 in promoting Treg differentiation has not been previously reported. Furthermore, induction of IRF3 activity by indole or other MDTMs, to our knowledge, have not previously been demonstrated. However, a dietary indole

derivative, indole-3-carbinol, has been shown to decrease IRF3 activation in RAW264.7 macrophages[282].

While our results do not confirm the direct contribution of IRF3 to indole-mediated augmentation of Treg differentiation, we were able to confirm the upregulation of multiple genes in the IRF3 regulatory pathway in indole-Tregs. *Ikbke* encodes IκB kinase ε (IKKε), which is involved in phosphorylating adaptor molecules that facilitate IRF3 activation and was upregulated 2.6-fold. Upon translocation to the nucleus, IRF3 binds to interferon-stimulated response elements upstream of target genes, including those of *Ifnb1* and many *Ifna* genes. We were able to confirm the upregulation of *Ifnb1* by 2.1-fold and IFN-α genes, using a pan-IFN- α primer, by 2.1-fold in indole-Tregs. Interestingly, IAld administration in a murine model of graft-vs-host disease (GVHD) upregulated type I interferon signaling and ameliorated disease progression[188]. Knockout of the IFN-α receptor ablated the protective effects of IAld, demonstrating dependence on type I interferons for inhibiting GVHD pathology. While the authors made no direct link to IRF3 activity, these results suggest immune tolerance-promoting effects of MDTMs may be dependent on induction of type I interfons.

In addition to IRF3, another transcription factor, NR4A3, was also predicted to be activated in indole-Tregs. In contrast to the novelty of the IRF3-Treg differentiation axis, NR4A transcription factors have been known to be directly involved in Treg differentiation[279]. Mice with NR4A1, NR4A2, and NR4A3 triple knockout were unable to produce thymic Tregs, while combinations of double knockouts also demonstrated impaired Treg development. The untargeted identification of a *bona fide* transcription

factor essential to Treg differentiation increases our confidence in the ability of our analysis methods to implicitly identify network regulators involved in T cell differentiation.

Amongst the top regulators found in 5-HI differentiated Th17 cells were LPS and TNF, two major molecules involved in pro-inflammatory signaling. This suggests that 5-HI may act similarly to a pathogen associated molecular pattern, driving Th17 differentiation and upregulating transcription of inflammatory cytokines. The most differentially regulated gene in 5-HI augmented Th17 cells, *Il22*, encodes IL-22, which along with IL-17 are the major Th17 cytokines. IL-22 has been identified at elevated levels in the colon of IBD patients and is also associate with inflammatory skin disorders like psoriasis[283-285]. When properly regulated, IL-22 contributes significantly to antimicrobial defense and maintenance of mucosal barriers through promoting epithelial repair and proliferation[286-288]. Interestingly, IAld administration in *Candida* infected mice induced IL-22 production that led to inhibition of Candidiasis[125]. IL-22 induction was found to be dependent on the AhR, which was also predicted to be activated in 5-HI Th17 cells (z-score = 2.6). Of note, *Il17a* was induced at a comparatively lower degree (1.86-fold as compared to 15.64-fold for *Il22*). This raises the possible therapeutic potential of 5-HI Th17 cells in situations where selective induction of IL-22 may be desired.

These results provide preliminary evidence with clear directions for future investigations. Activation of IRF3 can be more directly measured with various methods to validate the results of the network analysis. If indeed there is increase IRF3

phosphorylation, dimerization, or nuclear translocation in indole-Tregs, a subsequent path of investigation would be to see whether IRF3 activation alone is sufficient to induce differentiation. Further investigation can also be directed towards down-stream targets of IRF3, such as type I interferons, as their involvement in MDTM signaling have been demonstrated in other systems. In the context of 5-HI Th17 cells, identification of commensals that are capable of 5-HI synthesis may highlight species or taxa that may be associated with inflammatory disease. These possibilities are further discussed in the Future work section. Together, these results present a role for MDTMs in the differentiation of naïve T cells and identify two compounds in indole and 5-HI that have reciprocal effects on Treg and Th17 populations. Regulation of gut microbial production of these compounds may be critical in maintaining balance between these two potent T cell subtypes and promoting immune tolerance and homeostasis. Furthermore, the distinct transcriptomic profiles of MDTM-augmented T cells along with greater levels targeted differentiation may be potential avenues of therapeutic benefit.

5.5. Materials and Methods

5.5.1. Mice

Female C57BL/6 mice, ~5 weeks old, were purchased from The Jackson Laboratory. Male B6-Foxp3^{EGFP} and B6-IL17a^{EGFP} mice were purchased from the Jackson Laboratory at ~7 weeks old. All mice were allowed to acclimate to local environments for a minimum of 1 week before use. Three Foxp3^{EGFP} and three

IL17a^{EGFP} mice were used for microarray experiments. For experiments involving WT mice, splenocytes and lymphocytes were either sourced from a single mouse or pooled from multiple depending on the cells required for experiments.

5.5.2. Flow cytometry

Spleen and mesenteric lymph nodes were isolated from freshly sacrificed mice. Tissues were dissociated between frosted glass slides and single cell suspensions were stained with αCD4-efluor450 (eBioscience clone GK1.5) and αCD25-PECy7 (eBioscience clone PC61.5) and Fc Block (BD Biosciences). $CD4^+CD25^-$ populations were sorted to >97% purity in a BD FACS Aria II cytometer and seeded in 96-well culture plates at a density of 10^5 cells per well.

For analysis of *ex vivo* differentiated cultures from WT mice, cells were stained with CD4 Monoclonal Antibody, eFluor 450 (eBioscience clone GK1.5) and CD25 Monoclonal Antibody, PE-Cyanine7, (eBioscience clone PC61.5), followed by incubation in Foxp3 fixation and permeabilization buffer (eBiosciences). Cells were then stained using αIL-17a-PE (eBioscience clone eBio17B7) or αFoxp3-APC (eBioscience clone FJK-16s) and analyzed in a BD Fortessa X-20 flow cytometer. Data were processed using FlowJo software (Tree Star, San Carlos, CA).

For isolation of RNA from pure populations of Tregs or Th17 cells, *ex vivo* differentiated cells from *Foxp3*-GFP OR *Il17a*-GFP mice were pooled from multiple wells for a given condition and sorted based on GFP fluorescence in a BD FACS Aria II

to a purity of >97%.

5.5.3. *Ex vivo* T cell differentiation

96-well round bottom (Falcon) plates were coated with 5 μg/mL αCD3 (BioXcell clone 145-2C11) and 2 μg/mL αCD28 (BioXcell clone 37.51) in PBS supplemented with calcium and magnesium ions overnight. Naïve T cells were seeded at a density of ~$1x10^5$ cells per well in RPMI 1640 supplemented with 2-mercaptoethanol, gentamicin, penicillin, streptomycin, and 10% FCS and were cultured at 37°C and 5% CO_2.

Media for Treg-skew experiments were supplemented with 2 ng/mL TGF-β (Peprotech) and 100 U/mL IL-2 (Roche). Media for Th17-skew experiments were supplemented with 10 ng/mL IL-6 (Peprotech), 5 ng/mL IL-23 (R&D), 0.5 ng/mL TGF-β (Peprotech), 10 μg/mL αIL-4 (BioXCell clone 11B11), and 10 μg/mL αIFN-γ (BioXCell clone R4-6A2). For Th17 experiments, cell cultures were stimulated with phorbol-12-myristate-13-acetate (50 ng/mL) and ionomycin (1 μM) for 4 hours before staining.

5.5.4. RNA isolation

Cells were washed with PBS and stored at -80°C until isolation. RNA were extracted using either EZNA Total RNA Kit (Omega Bio-Tek) with Omega Homogenizer columns or RNeasy Micro Kit (Qiagen) with QIAShredder columns and on-column DNAse digestion.

5.5.5. Microarray and network signaling analysis

RNA samples represent pure populations of Tregs of Th17 cells cultured with or without MDTMs from the spleen and mesenteric lymph nodes of three separate transgenic mice per condition. The samples were analyzed on an Affymetrix Mouse Transcriptome Assay 1.0 chip (now GeneChip Mouse Exon 1.0 ST Array) at the Genomics and Microarray Core at UT Southwestern. Raw files were imported to the Transcriptome Analysis Console 4.0 (ThermoFisher) for gene- and exon-level analysis as biologically paired data with three replicates. Relative gene expression levels were calculated based on signal space transformation and robust multi-array averaging, including GC Correction version 4. Genes with a differential gene expression > 2-fold, p-value < 0.5, were used as inputs in Ingenuity Pathway Analysis (Qiagen). Upstream regulator analysis was used to identify enriched regulatory nodes.

5.5.6. Quantitative reverse transcription PCR

Total RNA was quantified in a Nanodrop 2000. qRT-PCR was performed using qScript One-Step SYBR Green reagents in a Lightcycler 96 System (Roche).

Primer pair sequences

Il13, FW	5' - AACATCACACAAGACCAGACTC-3'
Il13, RV	5' - GGGCCTTGCGGTTACAGA-3'
Cxcl9, FW	5' - ATCATTGCTACACTGAAGAACGG-3'
Cxcl9, RV	5' - TCTTATGTAGTCTTCCTTGAACGAC-3'
Nr4A1, FW	5' - CTTCAAGCGCACAGTACAGAAAA-3'
Nr4A1, RV	5' - GAGGCTGCTTGGGTTTTGA-3'
Irf3, FW	5' - GACAAAGAAGGGGGTTGCG-3'
Irf3, RV	5' - GGGCAAAATCCGCGGTTT-3'
Ifnb1, FW	5' - GTCCTCAACTGCTCTCCACT-3'
Ifnb1, RV	5' - TGCAACCACCACTCATTCTG-3'
Isg15, FW	5' - TCTGACTGTGAGAGCAAGCAG-3'
Isg15, RV	5' - ACGGACACCAGGAAATCGT-3'
Ikbke, FW	5' - TGGAGTGCAGGAAGAAGATAGC-3'
Ikbke, RV	5' - CTCAGGAGGTCGCCGATAG-3'
Irf7, FW	5' - TCCGCATAAGGTGTACGAACT-3'
Irf7, RV	5' - GTCCCCGGCATCACTAGAAA-3'
Nr4a3, FW	5' - CTTCAAGAGAACGGTGCAGAAAA-3'
Nr4a3, RV	5' - TGGAAGGCAGACGACCTC-3'
Ifna, FW	5' - CCTGCTGGCTGTGAGGAA-3'
Ifna, RV	5' - TGCTCTGACCACCTCCCA-3'

6. CONCLUSIONS

6.1. Summary

MDTM have been shown to have pleiotropic functions in various immune contexts[39,45,46,49,131,188,289]. Our work set out to further characterize immunomodulatory effects of indole on macrophages, epithelial cells, and T cells, while also developing mechanistic insights into their functions. We identify indole as a potent inhibitor of TCDD mediated AhR signaling in colon epithelial cell lines. Yet, despite the relatively low expression of AhR and no classical induction of *Cyp1a1* gene expression in macrophages, we observe anti-inflammatory effects of MDTMs in these cells, suggesting the presence of cell-type specific signaling mechanisms. Indeed, we find chemical inhibition of the AhR and comparisons with $AhR^{-/-}$ macrophages to not have significant effects on indole-mediated reduction of TNF accumulation.

Gathering further mechanistic insights into the anti-inflammatory signaling of indole in macrophages, we employ a battery of chemical inhibitors to probe dependency on multiple pathways. Inhibition of PI3K, AKT, p38 MAPK, and JNK failed to ablate reduction of TNF accumulation in RAW264.7 macrophages, suggesting mechanisms independent of these pathways. Furthermore, while we observed decrease in TNF accumulation with indole pretreatment of macrophages, a concomitant reduction in *Tnf* mRNA levels was not observed, despite testing of multiple time points. To test the possibility of post-transcriptional regulation of TNF protein levels through degradation pathways, we utilize the chemical proteasomal and lysosomal inhibitors, MG-132 and

bafilomycin, respectively. Neither inhibitor ablated indole-mediated reductions in TNF protein accumulation, suggesting a degradation-independent mechanism.

Another level of gene/protein regulation that may fit our pattern of observations is in that of miRNA-mediated inhibition of mRNA translation. Previous studies have demonstrated miRNA transfection decrease protein levels of cytokines, such as TNF and IL-6, without significantly altering mRNA expression levels[207,208]. In particular, miR-181b and miR-124 have been shown to exhibit these effects in RAW264.7 macrophages. We sought to quantify changes in the expression of these miRNAs in indole pretreated cells but failed to observe any significant upregulation. Our work in this area narrows down the potential pathways in which indole my signal through to exert its anti-inflammatory effects in macrophages, while highlighting cell-context dependency in pathway signaling, as the AhR-mediated effects in epithelial cells are not recapitulated. While we were unable to identify a specific mechanism facilitating indole's effects, our work provides rationale for further investigations into a miRNA-MDTM signaling axis.

In an alternative approach, we synthesize a novel cell-impermeant molecule with an indole moiety bound through a flexible aliphatic linker to BSA. We posit that this molecule, I3B, limits indole's interactions with cells to surface-level mediated effects. We observe a 50-fold potentiation of the anti-inflammatory effects of indole after BSA linkage, suggesting the presence of a cell-surface receptor that may facilitate potent anti-inflammatory signaling. Transcriptome profiling and subsequent experiments reveal I3B functions in part through the cytosolic transcription factor, NRF2. A major downstream target of NRF2, *Hmox1*, has previously been identified as a mediator of IAA anti-

inflammatory effects in macrophages. However, to our knowledge, this is the first identification of NRF2 as being a mediator of MDTM function downstream of cell surface interactions.

Beyond macrophages and epithelial cells, we identify two MDTMs, indole and 5-HI, as having reciprocal effects on the differentiation of naïve T cells into Tregs and Th17 cells. Transcriptome profiling suggests activation of IRF3 and IRF7 transcription factors in indole augmented Tregs. While some downstream targets of IRF transcription, particularly type I interferons, have been found to be involved in MDTM signaling in astrocytes and in the context of graft-vs-host disease, to our knowledge, this is the first identification of IRFs themselves as a potential regulators of indole function[42,188]. 5-HI augmented Th17, on the other hand, display a generally pro-inflammatory transcriptome profile, with patterns indicative of increased signaling in the TNF and LPS signaling pathways, with particularly high levels of *Il22* induction.

6.2. Future Directions

While our work identifies NRF2 as a critical transcription factor involved in facilitating the anti-inflammatory effects of I3B on macrophages, detailed mechanisms are yet to be elucidated. As NRF2 is a cytosolic transcription factor, direct interaction between I3B and the protein is unlikely. This leaves significant room for further investigation. We posit that I3B engages a cell surface receptor that, in turn, stimulates NRF2 activation. However, this receptor is yet to be identified and should be the next step in this work.

The potentiation of indole's anti-inflammatory effects on macrophages by I3B is an interesting result; however, most of our investigations have been limited to this cell type, along with some work in epithelial cells. Numerous aspects of our work demonstrate the existence of cell-context dependent effects, and indeed, I3B affects AhR signaling in epithelial cells, while similar results could not be found in macrophages. While we have identified NRF2 as a critical transcription factor in macrophages for I3B function, whether this mechanism extends to epithelial cells is yet to be seen. Our results make future investigations into signaling axis a promising target for further investigation. Beyond this, the effects of I3B on other immune cell types such as T cells and dendritic cells remain unexplored. With evidence that T cells and hepatocytes are responsive to indole treatment, it stands to reason that I3B may also have potentiated effects here and warrants investigation.

While I3B significantly upregulates NRF2 target genes in macrophages, the same is not observed for free indole, suggesting cell surface interaction of these molecules induces a distinct signaling profile from intracellular interactions. In our work, we eliminate some possible mechanisms of indole signaling, but have yet to identify a clear mechanistic target for free indole. However, our work suggests miR-mediated inhibition of inflammation may be a critical mechanism for indole signaling. While we were unable to identify miR targets upregulated by indole, high-throughput screening methods may be employed to find what we could not with our limited targeted investigations.

Our work in T cells identify IRF3 and IRF7 as potential regulators of indole mediated Treg differentiation in naïve T cells. We identify gene transcription signatures

consistent with IRF3/IRF7 activation, but further validation of this hypothesis through the direct observation of IRF3 phosphorylation or dimerization in indole treated Tregs would support our findings. Furthermore, the use of either genetic knockout mice or neutralizing antibodies may be instrumental in investigating whether indole-mediated type I interferon signaling is tantamount to its augmentation of Treg differentiation.

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