IDENTIFICATION OF THE MICROBIOTA METABOLITE, INDOLE, AS A NOVEL IMMUNE MODULATOR FOR ANTIGEN-PRESENTING CELLS IN THE GUT

A Dissertation

by

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ABSTRACT

Mammals are colonized by trillions of symbiotic microbes, termed the microbiota. This collection of predominantly bacteria greatly outnumbers the host's own cells in number, genomic content, and biochemical potential. Extensive research has revealed the necessity of the microbiota for developing a fully functional intestinal and systemic immune system. While definitive crosstalk between host microbiota and immune system exists, the discrete compounds responsible for altering immune cell function remain to be fully characterized. Antigen-presenting cells (APCs) are fundamental regulators of immunity and integrate signals from their local environment to direct immune responses. Primary roles of APCs include antigen presentation to naïve T cells, tissue integrity maintenance, and cytokine secretion to instruct activity of other immune cells. APCs residing at mucosal sites have a unique role in maintaining homeostasis by promoting peripheral tolerance to harmless commensal microorganisms. The dysregulation of this phenomenon promotes chronic inflammation in the intestinal tract, which predisposes the host to numerous cancers and metabolic disorders. Identifying and manipulating the specific microbiota components that drive tolerance in the gastrointestinal tract is a primary goal of current immunological research.

Indole is a microbiota-derived metabolite produced by numerous bacterial species and present at high concentrations in the intestines. Previous work from our lab and others has demonstrated a protective role of indole in the GI tract. In this study, we show that

indole is able to suppress pro-inflammatory responses and promote mucosal phenotype and function in APCs. Remarkably, indole-conditioned dendritic cells (DCs) imprinted naïve T cells with gut-homing markers and preferentially induced regulatory T cells. Our overall findings reveal that indole conditions DCs towards a mucosal phenotype in a manner mechanistically distinct from the canonical GI signal, retinoic acid. In addition, indole-conditioned DCs are capable of promoting a regulatory phenotype in naïve T cells. These observations reveal a novel mechanism by which an endogenous microbiota metabolite conditions APCs for optimal function in mucosal tissues, thus providing evidence for a single metabolite promoting properties associated with peripheral tolerance. This revelation paves the way for future work in manipulating the microbiota for therapeutic potential in autoimmune and inflammatory disorders of the GI tract.

DEDICATION

In loving memory of my Uncle Errol, the bravest man I have ever known.

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My family is my base of support, and I hope someday we might all live within a few states of one another. My parents and my sister, Kristina, have offered nothing but encouragement and support from day one of this journey and for this I will never be able to thank them enough.

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NOMENCLATURE

APC	Antigen-presenting cell
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophage
TLR	Toll-like receptor
LPS	Lipopolysaccharide
CpG	CpG oligodeoxynucleotide
HK-STM	Heat-killed *Salmonella typhimurium*
RA	Retinoic acid
Treg	Regulatory CD4+ T helper cell
Th1	CD4+ T helper type 1
Th2	CD4+ T helper type 2
Th17	CD4+ T helper type 17
AhR	Aryl hydrocarbon receptor

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The anatomy and organization of the gut-associated lymphoid tissue (GALT)

Of all the anatomical locations of the human body, the immune tissues associated with the gastrointestinal (GI) tract are perhaps charged with the most difficult task: to maintain a hyporesponsive tolerance towards the trillions of commensal microorganisms and environmental antigens to which it is constantly exposed, while remaining poised to initiate appropriate inflammatory responses against invading pathogenic organisms. Most human pathogens enter the body via mucosal surfaces, including the intestines (1), thus a strong and accurate immune response is crucial to prevent dissemination of infection. Meanwhile, inappropriate activation of immunity against harmless bacteria results in tissue damage and chronic inflammation, which are detrimental to the host and increase predisposition towards a multitude of inflammatory disorders. The delicate balance between immune tolerance and activation is achieved in a variety of ways that make the intestines a truly unique immunological site.

The organization of intestinal immune components is uniquely suited to support these opposing functions. A single layer of simple columnar epithelial cells lines the intestinal tract, separating the immense bacterial load in the intestinal lumen from the underlying host tissues. Tight junctions and adherens junctions assist in regulating epithelial

permeability and seal the paracellular space between cells (2). Intestinal goblet cells secrete mucins, forming a two-layered mucus barrier that largely prevents molecules and bacteria from contacting the epithelial layer (3).

Despite these mechanisms, immune cells contact the intestinal lumen environment in a number of ways. Intraepithelial lymphocytes, predominantly CD8+ T cells expressing αβ or γδ T cell receptors, are embedded within the epithelial layer (4). In addition, dendritic cells and macrophages have a unique ability to extend their dendrites between epithelial cells, enabling antigenic sampling across the epithelium. The expression of tight junction proteins by these specialized APCs allows the epithelial integrity to be maintained (5). A principal component of peripheral tolerance in the GI tract is the regulatory T cell (Treg), which actively suppresses effector T cells and prevents tissue damage from excessive inflammatory responses (6).

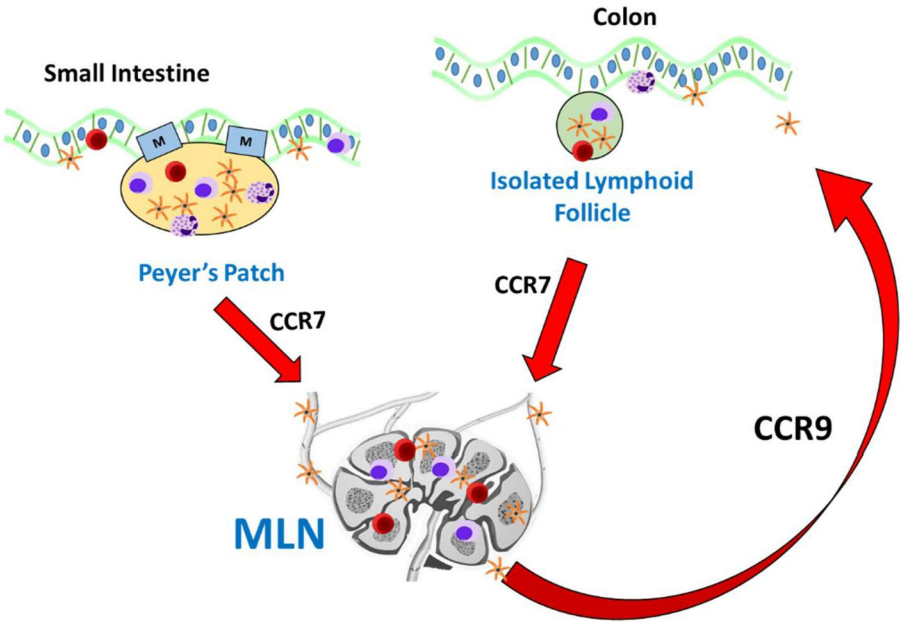

Figure 1. Anatomy and organization of gut-associated lymphoid tissue (GALT).

Organized lymphoid tissues are present throughout the gut. Peyer's patches are lymph node-like structures located along the small intestine, whereas isolated lymphoid follicles are distributed in both the small and large intestines. Specialized "M" cells facilitate transcytosis of antigens across the epithelial surface. Numerous immune cells, including B cells, T cells, dendritic cells, and macrophages reside within these tissues. Immune cell trafficking to mesenteric lymph nodes via the lymphatic system is mediated by expression of the chemokine receptor, CCR7. Activated immune cells can be redirected back to the intestinal microenvironment by expression of chemokine receptor, CCR9.

Much of the gut-associated lymphoid tissue (GALT) is organized into distinct tissues: the mesenteric lymph nodes (MLNs), Peyer's patches (PPs) and isolated lymphoid follicles (ILFs; Figure 1). Lymph nodes are critical initiation sites for adaptive immune responses against pathogens and are where dendritic cells present antigen to naïve T cells. It was discovered over a century ago that oral administration of a protein results in "immunologic unresponsiveness" upon systemic challenge using that same antigen (7). This phenomenon known as oral tolerance is essential for preventing aberrant immune responses against innocuous dietary antigens. An elegant study by Worbs *et al*. demonstrated that the MLNs are the exclusive site of oral tolerance induction and that this process relies upon antigen-loaded DCs migrating from the intestines to the MLNs (8).

Peyer's patches are lymph node-like structures located along the small intestine that are important for facilitating immune responses in the gut. Peyer's patches have a distinct dome-like appearance, containing numerous B cell lymphoid follicles (approximately 10 in mice and several hundred in humans) surrounded by smaller T cell areas and dendritic cells in the subepithelial dome (9). Distinct from lymph nodes, Peyer's patches lack an outer capsule and have persistent germinal centers, likely indicative of continual immune stimulation by luminal antigens (10). A follicle-associated epithelium separates these immune cells from the gut lumen and contains microfold, or M cells, specialized in transporting luminal vesicle-bound antigens across the epithelial surface and released in

the extracellular space below (transcytosis) for phagocytosis by dendritic cells. An important role of Peyer's patches is serving as the primary source of IgA-secreting plasmablasts that home to the small intestine (11). IgA is the dominant class of antibody in the mucosal system, secreted into the intestinal lumen at a rate of approximately 3 grams per day in humans (12). This secretory IgA binds to the mucus layer, which lines the epithelial surface, serving a crucial protective role by preventing adherence of bacteria as well as neutralizing toxins.

Isolated lymphoid follicles (ILFs) are similar in structure to Peyer's patches and distributed in both the large and small intestines. These lymphoid aggregates also rely upon M cells for transcytosis of luminal antigen to the immune cells below, however ILFs are mainly composed of B cells (13). Both Peyer's patches and ILFs are connected via the lymphatic system to the draining MLNs, thus allowing for migration and associated interaction of immune cells amongst these gut-associated lymphoid tissues.

Nearly 80% of an individual's immune cells belong to the mucosal-associated immune system, with the majority of these cells residing in the GI tract (14). This distribution of the immune system in extremely close proximity to trillions of bacterial antigens is not coincidental, and emerging evidence continues to reveal the vital importance of immune cell-microbiota crosstalk for optimal host immunity.

The microbiota is essential for proper immune system development and function

The trillions of symbiotic microorganisms that colonize a host are termed the microbiota. The most prevalent microbes are bacteria, reaching concentrations of 10^{12} cells per gram of luminal content in the colon and outnumbering the host's own genomic content by a factor of 200 (15). These commensal organisms have co-evolved with the host over time, having established a symbiotic relationship in order to promote homeostasis and prevent elimination by the immune system. Numerous beneficial effects of the microbiota have long been recognized, including colonization resistance against pathogens, (16) synthesis of vitamin K and most B vitamins (17), and fermentation of fiber to produce metabolically active products (18). While current research is drastically expanding our appreciation for the role of the microbiota in shaping health and disease by metabolic, immune, and additional means (19), the mechanism by which the microbiota regulates host immunity largely remains a "black box". Metabolites produced by the microbiota have emerged as a major possible pathway for mediating these effects.

Elegant studies utilizing germ-free mice have demonstrated the necessity of the microbiota for proper development of a fully functional intestinal and systemic immune system, with sterile animals exhibiting intestinal tissue defects, reduced nutritional and endocrine function, and high susceptibility to infection (20-22). Above all, both GI and systemic immune responses are impaired without the presence of commensal bacteria (23). It is evident that special adaptations in the immune system and its regulation,

particularly in the gut mucosa, have evolved to allow for a symbiotic co-existence with the microbiota.

Conserved pattern recognition receptors (PRRs) on the surface of innate immune cells recognize pathogen-associated molecular patterns (PAMPs), initiating immune responses upon detection of non-self molecular motifs. Dendritic cells and macrophages express all four identified classes of PRRs, including transmembrane-spanning Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic NOD-like receptors (NLRs) and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs). A crucial distinction by the host immune system must be made to determine whether the microorganism is a harmless commensal to be tolerated or a potential pathogen to which immunity should be activated. The distinct mechanisms that mediate this differentiation remain largely obscure, but substantial evidence points to local signals in the intestinal microenvironment mediated by APCs (discussed below).

Dysbiosis in the GI tract linked to inflammatory conditions

The gastrointestinal tract is the predominant site of interaction between the host immune system and microbiota. Imbalances in the composition of the microbiota, termed dysbiosis, disrupt the mutually beneficial relationship between microbiota and host immune system. Dysbiosis typically manifests as a change in microbiota composition and reduction in diversity. This disturbance promotes aberrant immune responses and chronic inflammation in the intestinal tract, predisposing the host to a wide array of

pathologies including colon cancer, inflammatory bowel disease, and metabolic disorders (24-26).

Inflammatory bowel disease (IBD), encompassing both ulcerative colitis (UC) and Crohn's Disease (CD), describes a group of related chronic inflammatory disorders of the GI tract with a steadily increasing global incidence (27). While genetic predisposing factors have been identified, it is well established that environmental factors play a substantial role in progression of the disease (28). The current dogma is that IBD is mediated by chronic inflammation in the GI tract likely resulting from inappropriate host immunity towards microbiota constituents (29). A likely driving factor for this condition is dysbiosis resulting from an imbalance of protective versus harmful bacteria (30, 31). Indeed, despite high variability amongst individuals, the microbiota composition of IBD patients is distinct from the healthy population (30, 32).

An interesting genetic comparison of colon biopsies from UC patients against their healthy twins revealed that UC is associated with lower biodiversity, unusual aerobic bacteria, and a greater proportion of bacteria of the *Proteobacteria* and *Actinobacteria* phyla (33), both of which are upregulated in IBD (26). A current challenge in this field is determining whether alterations to the microbiota are a cause of IBD versus a secondary phenomenon. For example, adherent-invasive *Escherichia coli* (AIEC) is well correlated with the ileum of Crohn's disease patients; however, whether outgrowth of this pathogen is a mediator of Crohn's disease or a secondary opportunistic pathogen

remains to be determined (34).

Conventional therapeutics for IBD including tumor necrosis factor (TNF) inhibitors, corticosteroids, and antibiotics are largely focused on suppressing inflammation in the GI tract. These options, in addition to surgical intervention, come with possible severe consequences and long-term adverse effects (35). Due to the chronic nature of IBD, therapeutics are administered long-term with the goal of maintaining remission and are accompanied by side effects including immune suppression, osteoporosis, steroid dependence, and an enhanced risk of active tuberculosis (36). Thus, the development of novel therapeutics for IBD is in great demand. Recent attention has been given to modulating the dysbiosis associated with IBD by means of bacterial therapy with the goal of repopulating the dysbiotic gut with protective commensal organisms. While probiotic and prebiotic administration have generally not been successful in attenuating IBD symptoms (37), the recent advent of fecal microbiota transplantation (FMT) appears to offer great promise (38). While well controlled studies are lacking, a systematic analysis of available FMT studies revealed that the majority of subjects have experienced an overall reduction in IBD symptoms and use of mediation as well as remission of disease and resolution of *C. difficile* infection (39). Despite the therapeutic potential suggested by early successes of microbiota transplant for suppressing IBD symptoms, numerous caveats prevent this from being a useful, mainstream therapeutic strategy. Safety is the ultimate concern when transferring live microorganisms into a recipient person. While screening of potential donors includes testing for known fecal

pathogens and transmittable diseases, there is a concern that undetected pathogens might be unintentionally transferred (38).

FMTs are impractical due to the frequency of administration required to maintain benefits, the unstable and potentially biohazardous nature of fecal material, and the impractical route of transfer, with available options consisting of nasogastric tube, upper tract endoscopy, colonoscopy, or retention enema (40). Given these constraints, the use of immune-modulating endogenous metabolites purified from the microbiota would represent a potentially valuable therapeutic approach, which circumvents current safety and practicality issues associated with FMTs. Thus, identifying and manipulating the specific microbiota components that drive tolerance in the GI tract is a primary goal of current immunological research.

Antigen-presenting cells in the gut maintain a unique, tolerogenic role

Among the multiple cell types present in the GI tract, antigen-presenting cells (APCs) are fundamental regulators of immunity that integrate signals from their local environment to direct immune responses in the context of antigen presentation to naïve T cells (Figure 2). APCs of the intestinal mononuclear phagocyte system have a unique role in maintaining homeostasis by promoting peripheral tolerance, a state of immune hyporesponsiveness towards harmless commensal microorganisms and nominal dietary components, which protects the host from aberrant inflammation. Indeed, these APCs are charged with discriminating harmful from harmless in an environment rich in immune-stimulating antigens including lipopolysaccharide, peptidoglycans, CpG-containing DNA, lipoproteins, and flagellin.

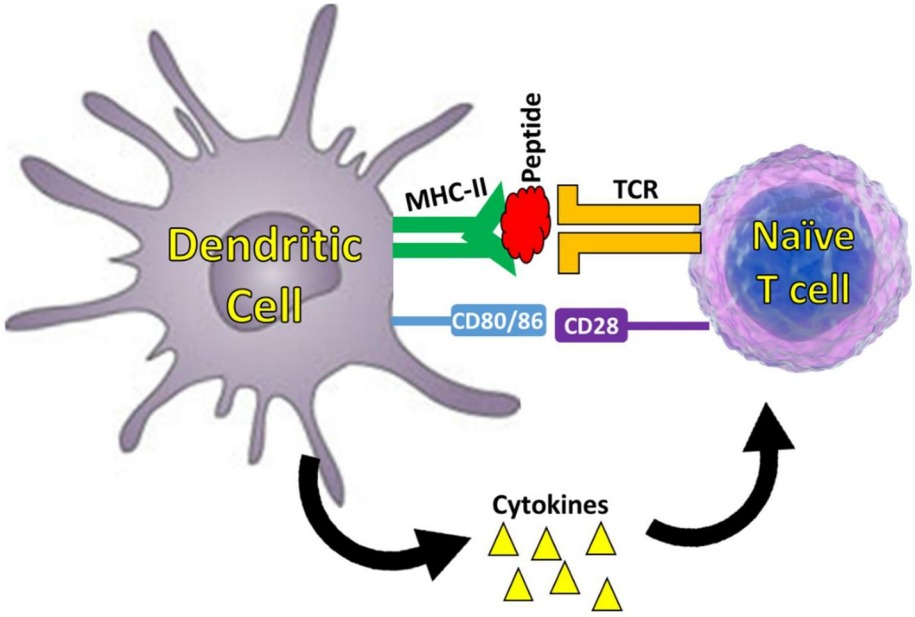

Figure 2. Mechanism of naïve T cell activation by a dendritic cell.

The activation of a naïve T cell by an antigen-loaded dendritic cell (DC) typically requires three signals. The first is interaction between the T cell receptor (TCR) and major histocompatibility complex (MHC)-peptide complex on the DC. The second signal is interaction of DC costimulatory markers (often CD80 or CD86) with CD28 co-receptor on the T cell. The third signal comes from the DC and can be soluble or cell contact-mediated. This is most commonly cytokine production by the DC, which interprets environmental signals and secretes appropriate cytokine signals to instruct the lineage fate of the newly activated T cell.

The two major classes of professional APCs, dendritic cells (DCs) and macrophages – each with specialized subtypes – have distinct yet complementary roles in regulating intestinal immunity. Both DCs and macrophages are found throughout the lamina propria as well as the Peyers' Patches, isolated lymphoid follicles, and related mucosa-associated lymphoid tissues (41). Activated DCs constitutively migrate from the lamina propria to the mesenteric lymph nodes, in a CCR7-dependent manner (42), where they present antigen to naïve T cells and secrete factors that polarize T cells toward various effector fates (43). These gut-associated lymphoid tissue (GALT) DCs possess unique properties including preferential induction of regulatory T cells, antigen sampling across the intestinal epithelium, induction of B-cell class switching to IgA, and promotion of gut-homing properties on lymphocytes (5, 44-46). This is in stark contrast to peripheral DCs in largely sterile tissues that migrate to draining lymph nodes and present the offending antigen to naïve T cells upon engagement of their pattern recognition receptors (e.g., TLRs). This antigen presentation by peripheral DCs typically primes the adaptive immune system to an effector Th1 or Th2 response for robust pathogen clearance. Through incompletely understood mechanisms, intestinal DCs constitutively produce retinoic acid, which drives mucosal DC-specific functions including Foxp3+ Treg induction, imprinting of gut-homing CCR9 on DCs and T cells, and B cell class switching to IgA (discussed below) (15).

In contrast to DCs, the primary function of macrophages is centered around local clearance of pathogens from the tissue. Additional functions include alerting other

immune cells of inflammatory insults as well as producing cytokines to maintain the local homeostatic environment and T cell reactivation (47). T cell-mediated colitis has been found to induce massive mesenteric lymph node recruitment of monocytes that differentiate to inflammatory macrophages and drive Th1 differentiation both *in vitro* and *in vivo* (48). An influx of newly differentiated macrophages is similarly observed in human IBD (49). While tolerogenic APCs appear crucial for gut homeostasis and most evidence points to a local conditioning on DC and macrophage precursors after arrival from circulation into the mucosa (50), the gut-specific signals that mediate the acquisition of these properties remain incompletely understood.

Known APC-modulating signals are present in the GI tract

Retinoic acid

While definitive crosstalk between host microbiota and immune system exists, the discrete compounds responsible for regulating immune cell function remain to be fully characterized. Relatively few gut-tropic immune modulators have been reported for APCs. Retinoic acid (RA), a metabolite produced by the host-mediated catabolism of dietary vitamin A, is perhaps the best-characterized signal in the gut. The precursor retinol and its chaperone, retinol-binding protein (RBP) are taken up into APCs via surface receptor STRA6 (51). Retinol is then reversibly oxidized to retinal by alcohol dehydrogenases, and the final irreversible step in retinoic acid production is mediated by intracellular retinaldehyde dehydrogenase (52)(Figure 3). Importantly, retinaldehyde dehydrogenase expression is tightly regulated at both the cellular and tissue level,

keeping retinoic production strictly controlled (53). This mechanism ensures that RA is produced by and biologically available to RA-responsive cells, including APCs.

RA is currently the only identified inducer of mucosal homing markers on DCs. The role of RA in imprinting gut homing markers on T cells has been elegantly demonstrated by a marked reduction in intestinal-dwelling T lymphocytes in vitamin A-deficient mice (53). In addition, RA appears to be a critical factor in mucosal DC-mediated generation of IgA+ B cells, as this function can be modulated by the antagonism or addition of exogenous RA (45). A role for RA in Foxp3+ Treg generation has been well demonstrated in the activation of naïve T cells by lamina propria-derived DCs (54, 55). Interestingly, it has been demonstrated that activation of primary DCs in the presence of RA can induce production of transforming growth factor-β (TGF-β) (56), revealing a mechanism by which components of the intestinal environment act in synergy to promote additional tolerogenic factors.

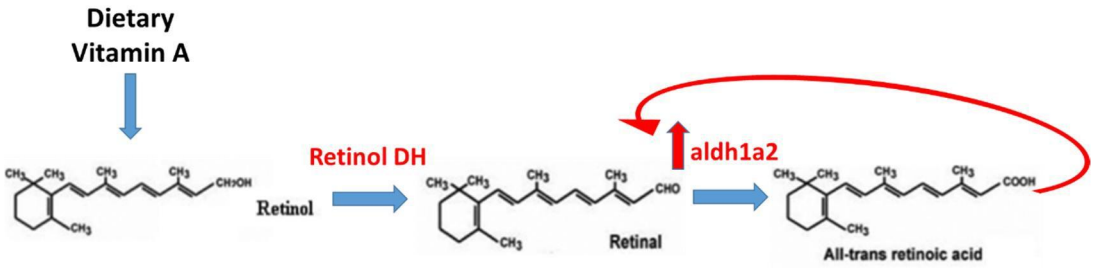

Figure 3. Metabolism of dietary vitamin A results in retinoic acid production.

Dietary vitamin A is converted to retinol in the small intestine, which is then taken up by cells including dendritic cells. Retinol is then converted to retinal by a series of alcohol dehydrogenases. The final reaction is catalyzed by aldehyde dehydrogenase 1a2 (aldh1a2), a tightly regulated enzyme which produces biologically active all-trans retinoic acid.

TGF-β

The cytokine, transforming growth factor-β (TGF-β) is another well characterized host-derived molecule present at high concentrations in the gut, produced by every leukocyte lineage including dendritic cells, macrophages, and lymphocytes (57). TGF-β has a predominant immunosuppressive role, acting by several mechanisms including inhibiting DC maturation and promoting the Treg lineage (55, 58, 59). Exposure to TGF-β during BMDC development inhibits subsequent antigen-presenting capacity, suggesting a means by which TGF-β dampens T cell activation in the intestinal environment (60). Mucosal DCs readily produce TGF-β both *in vitro* and *in vivo* (58), and TGF-β has been identified as an essential co-factor in MLN-derived DC induction of Foxp3+ Tregs (55).

Several other molecules of host-derived origin, including IL-10, vasoactive intestinal peptide, and thymic stromal lymphopoetin have been reported to modulate intestinal DC maturation and function (61, 62). Additional signals are produced by bacterial components of the microbiota and can modulate APCs. Of these microbiota-derived signals, *Bacteroides fragilis* polysaccharide A (PSA) and short-chain fatty acids (SCFAs, discussed below) are the most fully characterized and both appear to promote tolerogenic properties in immune cells. However, millions of bioactive microbiota products remain to be characterized. Thus, the identification of additional microbiota-derived factors affecting APC phenotype and function in the gut would provide

important information on the functional mechanisms by which the microbiota regulates intestinal immunity.

Some microbiota metabolites have been identified as immunomodulators

Short-chain fatty acids (SCFAs)

While a few microbiota products have been reported to influence host physiology and immunity (Figure 4), the compounds responsible for microbiota control of host immunity are largely unknown. Receiving the most attention are the SCFAs, characterized decades ago as bacterial fermentation products of dietary fiber. This class of metabolites, typified by its predominant members butyrate, propionate, and acetate, are produced by Firmicutes and Bacteroidetes bacterial strains at millimolar levels in the human colon (63). Molecular characterization of these SCFAs indicates that they function as histone deacetylase (HDAC) inhibitors and ligands for G protein-coupled receptors in immune cells (64, 65). The clinical relevance of SCFA therapy in the GI tract has been established, with some IBD patients receiving benefit from SCFA-containing enemas (66). Indeed most studies have focused on the direct effects of SCFAs in the colon, demonstrating a preferential induction of regulatory T cells which is mediated by increased Treg gene expression and suppressive capacity in a GPR43-dependent manner (67-69).

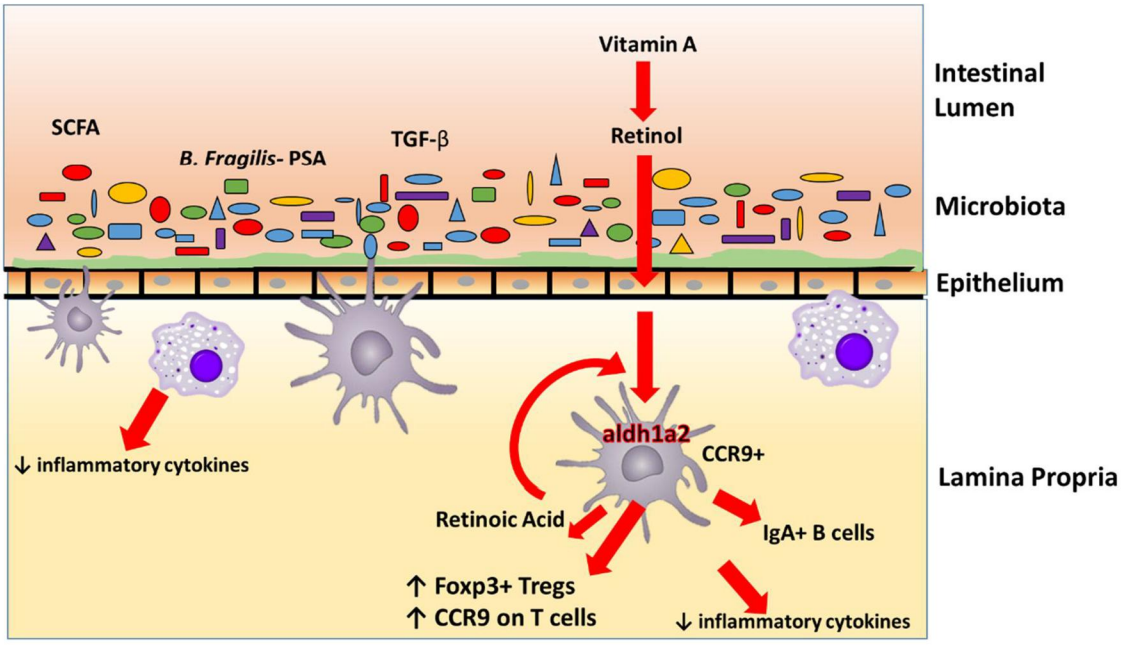

Figure 4. Known host and microbiota mechanistic regulators of intestinal APCs.

Known gut-centric molecules which induce tolerogenic APCs include microbiota-derived short-chain fatty acids (SCFA) and *Bacteroides fragilis* with its outer membrane component, polysaccharide A (*B. fragilis*- PSA). The established, predominant host- derived factors which drive tolerogenic DC properties are the cytokine transforming growth factor-β (TGF-β) and the dietary vitamin A metabolite, retinoic acid (RA). Mucosal DCs express high levels of the enzyme aldehyde dehydrogenase 1a2 (aldh1a2) which catalyzes the RA-producing reaction, thus producing high concentrations of RA in the microenvironment. This RA acts directly upon DCs and CD4+ T cells to affect gene transcription for downstream effects including preferential induction of Foxp3+ Tregs, expression of gut-homing CCR9 receptors, class switching to IgA+ in B cells, and suppressed inflammatory responses to TLR stimuli.

Immune modulating effects of the SCFA, butyrate, have also been reported in bone marrow-derived macrophages including inhibition of HDACs. This inhibitory activity led to an increase in histone 3 lysine 9 acetylation (H3K9Ac) at the promotor regions of *IL6*, *IL12b*, and *Nos2*, resulting in decreased production of pro-inflammatory IL-6, IL-12 and nitric oxide (70). These anti-inflammatory responses were also observed in primary lamina propria-isolated macrophages; however, butyrate-supplemented drinking water was not able to ameliorate DSS-induced colitis (70). While the reason for this failure of protection is unknown, it should be noted that the DSS-treated mice were also administered daily oral antibiotics to deplete butyrate-producing bacteria, thereby drastically altering the microbiota and undoubtedly producing off-target effects.

Polysaccharide A from Bacteroides fragilis

The capsular polysaccharide A (PSA) of the commensal bacteria *B. fragilis* has gained recent attention as an immune-modulating microbiota product. An impressive study utilizing the CD4+CD45Rb transfer model of colitis demonstrated that wild-type but not a PSA-deficient strain of *B. fragilis* is able to protect against experimental colitis, thereby identifying PSA as the essential immune-modulating component (71). Subsequent work by the same group demonstrated that monocolonization of germ-free animals with *B. fragilis* efficiently suppressed inflammation by inducing IL-10-producing Foxp3+ Tregs in the gut in a PSA-dependent manner (72). Ultimately, the studies revealed the mechanism that requires TLR2 recognition by DCs of PSA packaged within outer membrane vesicles (73). Intriguingly, TLR2 expression on T cells

is insufficient to mediate this effect (73). This compelling finding demonstrates a biological situation in which antigen-presenting cells are required for sensing a microbial product and subsequently modulating downstream T cell activation and function. We predict that other microbiota metabolites are similarly capable of instructing APCs in their interactions with other immune cells.

Indole

Indole is a strictly microbiota-derived metabolite produced from tryptophan by numerous Gram-positive and Gram-negative bacterial species that express the tryptophan lyase enzyme, such as *Escherichia coli* (74, 75). Already well established as an inter- and intra-species bacterial communication signal (75-78), more recent work has suggested that indole may also act as an inter-kingdom signaling molecule (74). Indole is freely diffusible and present in human and mouse luminal contents at concentrations reaching millimolar levels (79, 80), indicating direct contact with immune cells of the GALT. We and others have reported immune modulating properties of this metabolite.

We first identified that indole strengthens epithelial barriers and reduces pro-inflammatory signaling in human intestinal epithelial cells (74). *In vivo* beneficial effects of indole have also been reported in germ-free mice, with indole-treated mice demonstrating increased resistance to dextran sodium sulfate-induced colitis (81). Interestingly, a beneficial effect of indole administration was also observed in specific pathogen-free (SPF) mice, demonstrating that indole promotes and strengthens the

intestinal epithelial barrier in both germ-free and SPF mice (81).

In agreement with these observations, we have recently demonstrated that indole supplementation reduces intestinal injury and inflammation induced by non-steroidal anti-inflammatory drugs (NSAIDs; (82)). Based on our work in intestinal epithelial cells we predict that indole's beneficial effects might be due to support of epithelial barrier function and dampening of pro-inflammatory signaling; however, future work will aim to elucidate the mechanisms by which indole attenuates damage in NSAID enteropathy. Overall, there is strong evidence to suggest that indole is a beneficial signal in the GI tract that merits further investigation in to cell type specificities and mechanism of action.

Aryl Hydrocarbon Receptor (AhR)

Our previous identification of indole as a ligand of the immune-modulating aryl hydrocarbon receptor (AhR) suggests a potential mechanism of action (83). The AhR is a ligand-activated transcription factor that binds exogenous and endogenous polycyclic aromatic hydrocarbon ligands, inducing nuclear translocation and binding to the AhR nuclear translocator (ARNT), followed by the AhR:ARNT complex binding the dioxin response element (DRE) DNA sequence and modifying subsequent gene transcription (84). Activation of the AhR mediates various cellular activities including response to environmental toxins, proliferation, differentiation, and cytokine secretion (85).

While first identified as a receptor for the industrial toxicant dioxin (84), a meaningful role for the AhR in shaping immune responses has been appreciated- particularly in the gut. AhR appears to regulate Th17 versus Treg induction and is crucial for the maintenance and function of innate lymphoid cells (ILCs) and intraepithelial lymphocytes (IELs) in the gut (85-87). The presence of AhR on innate immune cells coupled with its ability to recognize environmental signals has led some to consider AhR as a potential pattern recognition receptor-like sensor for nutritional or metabolic-driven immune responses (88).

The specific function of AhR in APCs is incompletely characterized and appears highly ligand dependent (85). A screening study was performed to query a panel of AhR ligands (6-formylindolo [3, 2-b] carbazole (FICZ), indole 3-carbinol (I3C), curcumin, quercetin, and ligand precursor tryptophan) on their abilities to affect BMDC characteristics (88). Interestingly, all included AhR ligands augmented LPS-induced pro-inflammatory cytokine production (88), which appears contradictory to the established immunosuppressive nature of AhR activity (89). In contrast, a recent article identifies immune modulation of DCs by AhR agonist, 4-n-nonylphenol, as the mechanism for Treg induction and immune suppression in their immune sensitization model (90). It has also been reported that BMDCs derived from AhR-deficient mice are less capable of inducing Foxp3+ Tregs, suggesting that AhR signaling in DCs is involved in Treg induction (91). These findings suggest that AhR is active in DCs and can affect their function, thus an investigation of DC- and macrophage-specific AhR activation by an

endogenous intestinal ligand would provide important knowledge in the area of microbiota-immunity crosstalk.

Based on our published data and the reports of indole as a homeostasis-promoting factor in the gut, the exposure of APCs to high indole concentrations in the intestines and the crucial role of APCs in peripheral tolerance, we hypothesize that the endogenous microbiota metabolite, indole, is a novel gut mucosa signal that promotes intestinal homeostasis by inducing a mucosal phenotype and function in APCs.

CHAPTER II

INDOLE DIRECTLY CONFERS MUCOSAL PROPERTIES UPON DENDRITIC CELLS

Overview

The factors responsible for conferring mucosal properties upon dendritic cells in the GALT remain incompletely characterized. Based on the abundance of indole in the lower gastrointestinal tract and the resulting direct proximity of indole to mucosal antigen-presenting cells, we hypothesized that indole acts as a signal for DCs that induces tolerogenic properties. This study begins by investigating the direct influence of indole conditioning on DC phenotype and function. The following findings emerged:

1. Indole selectively inhibits pro-inflammatory cytokine and signaling pathways in dendritic cells in an AhR-independent manner.
2. Indole does not alter antigen-presenting cell properties of dendritic cells.
3. Indole induces homing markers on dendritic cells.

Rationale

The majority of gut-tropic immune modulators that have been reported are derived from the host. Retinoic acid and TGF-β have distinct roles in shaping antigen-presenting cells for mucosal function; however, neither of these signals is responsible for imprinting dendritic cells with all of the tolerogenic properties observed in the gut. Intestinal

inflammation results in a loss of tolerogenic properties of mucosal DCs that cannot be rescued by adding endogenous RA to the system (92). This observation implicates additional mechanisms responsible for local conditioning of mucosal dendritic cells.

One of the putative signaling molecules is indole. This microbiota-derived metabolite is freely diffusible and present in the gut lumen of humans and mice at concentrations reaching millimolar levels (79, 80), indicating direct contact with immune cells of the lamina propria that sample the luminal contents. Furthermore, we have previously demonstrated that indole strengthens epithelial barriers and reduces pro-inflammatory signaling in human intestinal epithelial cells (74), suggesting a role for indole in maintaining GI tract tolerance. The beneficial effects of indole have also been demonstrated in both germ-free and conventional mice where indole-treated mice displayed increased resistance to dextran sodium sulfate-induced colitis; however, the possible contribution of antigen-presenting cells in this model was not addressed (81). Intriguingly, fecal levels of indole are reduced in UC patients (93), suggesting reduced indole production by the inflamed microbiota. Collectively these findings lead us to the hypothesis that the dysbiosis associated with colitis favors non-indole producing bacteria, and the absence of local DC conditioning by indole contributes to the observed loss of tolerogenic DC properties. Thus, we set out to determine whether indole might promote a tolerogenic phenotype and function in dendritic cells.

Results

Indole selectively inhibits pro-inflammatory cytokine and signaling pathways in dendritic cells in an AhR-independent manner

Pro-inflammatory cytokines

One important way that DCs shape local immune responses is through the production of cytokines. Because we had previously observed that indole dampens the gene expression of pro-inflammatory cytokines in intestinal epithelial cells (74), we first addressed cytokine production in indole-conditioned dendritic cells. We began our query in the murine dendritic cell line, DC2.4. As predicted, cells pre-treated with physiologically relevant doses of indole produced less TNF upon stimulation with the bacterial surface component and TLR4 ligand, LPS. Production of TNF was reduced by 50% following treatment with 1 mM indole (Figure 5). This response is biologically important as LPS serves as the prototypical endotoxin, present on the surface of virtually all gram-negative bacteria including gut-resident commensal strains. Thus, our results demonstrate the ability of indole to tolerize DCs against a relevant, inflammatory bacterial signal.

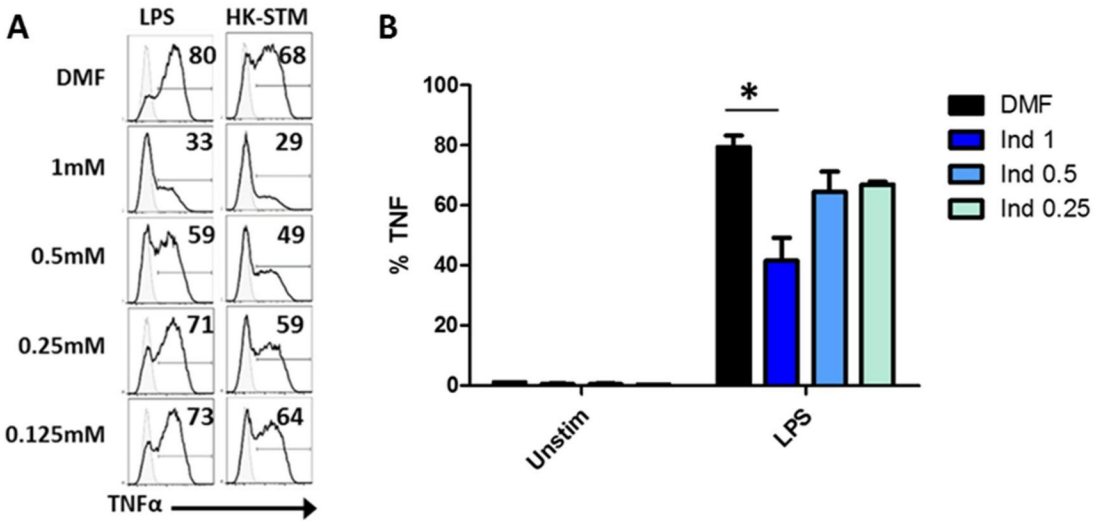

Figure 5. **Overnight indole treatment inhibits TNF production in DC2.4 cell line.**

Flow cytometric analysis of DC2.4 cells treated with indole for 24 hours and stimulated with LPS (1 ug/ml) or HK-STM (2 x 10^5 CFU) for 4 hours in the presence of Golgiplug protein transport inhibitor. Intracellular staining was performed to assess TNF production. (A) Representative histograms where shaded gray areas represent unstimulated samples. (B) Mean of three independent experiments. * $p < 0.05$ by Student's *t* test comparing indole-treated DCs to solvent control (DMF).

We then generated bone marrow-derived DCs (BMDCs) as described previously (94)(Illustrated in Figure 6). Briefly, bone marrow was harvested from femurs of mice and cultured for 7 days in the presence of DC-skewing cytokines, GM-CSF and IL-4. Treatment of these cultures with indole for 18 hours followed by TLR stimulation resulted in a similar inhibition of TNF production, reaching a magnitude of nearly 2-fold inhibition (Figure 7). We also queried the pro-inflammatory cytokines, IL-6 and IL-12 and found 2-fold or greater inhibition of cytokine production with overnight indole treatment (Figure 8). Immune cells communicate largely through the production of soluble factors including cytokines, and DCs detect these signals and determine their effector functions accordingly. Thus, the inhibition of pro-inflammatory cytokine production induced by indole conditioning would suggest that indole dampens DC responses to immunogenic signals, providing a potential mechanism by which mucosal DCs "ignore" harmless commensal bacteria in the gut.

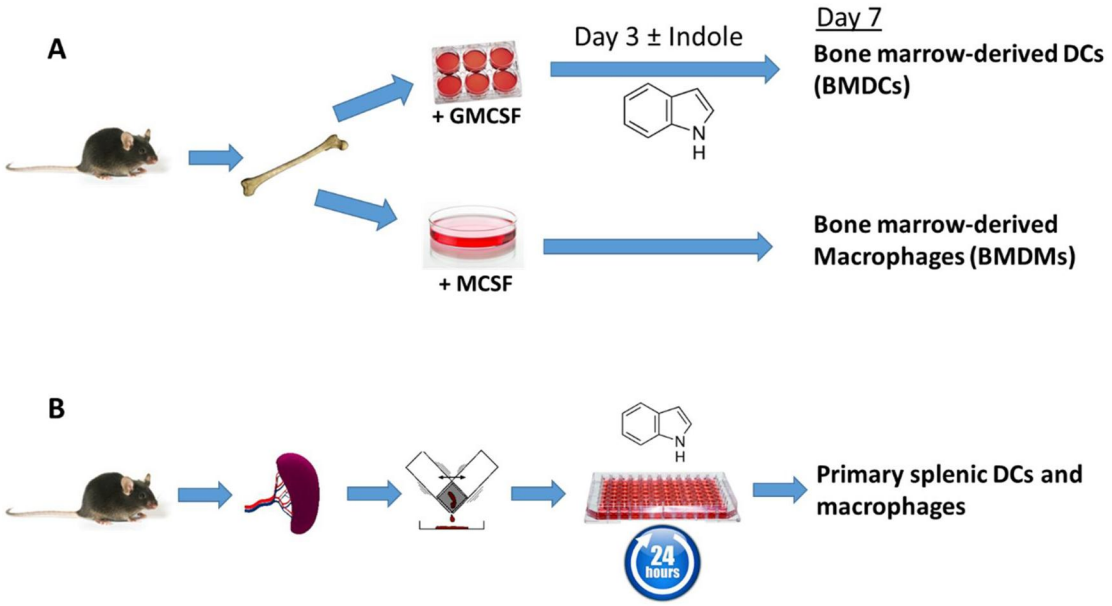

Figure 6. Culture methods used for bone marrow-derived and splenic APCs.

(A) Bone marrow was harvested from femurs of C57BL/6 mice and cultured for 7 days. BMDC cultures were supplemented with 20 ng/ml GMCSF and 10 ng/ml IL-4 with media replenished on days 2, 4, and 6. In some experiments, indole was added to the culture from day 3. BMDM cultures were supplemented with 10 ng/ml MCSF with media replenished on day 3. (B) Spleens were harvested from C57BL/6 mice and homogenized between 2 frosted microscope slides. Heterogeneous splenocytes were plated for 24 hours in the presence or absence of indole prior to TLR stimulation. Cultures were stained and splenic DCs and macrophages were identified by lineage surface markers using flow cytometry.

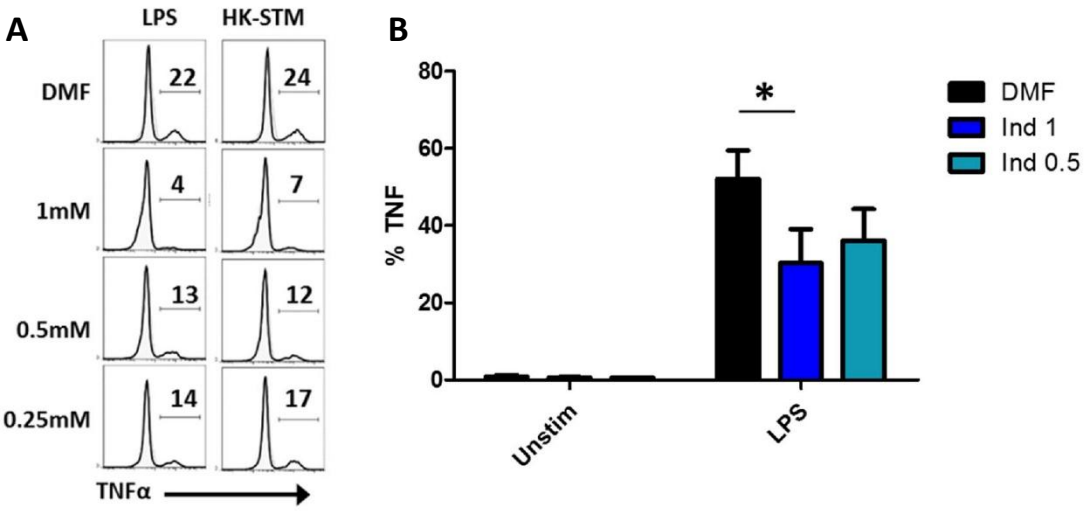

Figure 7. Overnight indole treatment inhibits TNF production in BMDCs.

Flow cytometric analysis of BMDCs treated with indole for 24 hours and stimulated with LPS (1 ug/ml) or HK-STM (10^7 CFU) for 4 hours in the presence of Golgiplug protein transport inhibitor. Intracellular staining was performed to assess TNF production. (A) Representative histograms where shaded gray areas represent unstimulated samples. (B) Mean of three independent experiments. * $p < 0.05$ by Student's *t* test comparing indole-treated DCs to solvent control (DMF).

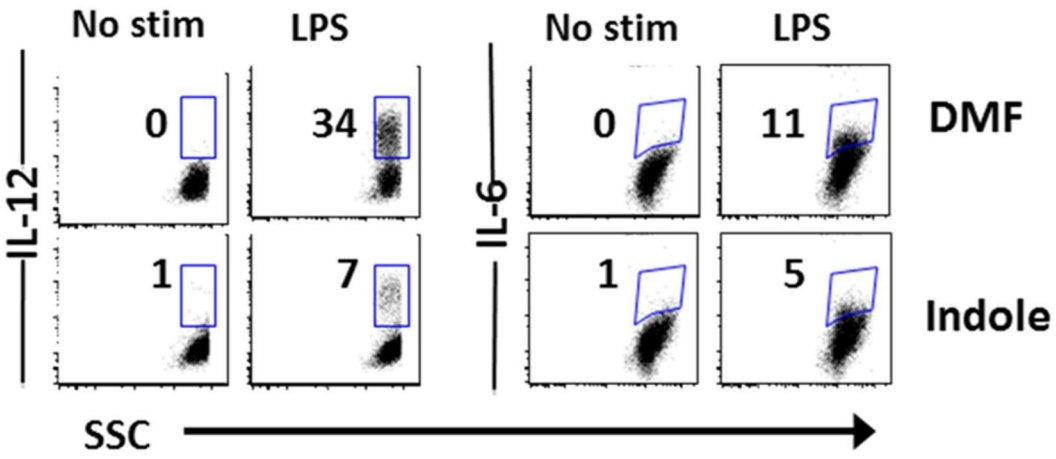

Figure 8. Overnight indole treatment inhibits IL-12 and IL-6 production in BMDCs.

Flow cytometric analysis of BMDCs treated with indole for 24 hours and stimulated with LPS (1 ug/ml) for 4 hours in the presence of Golgiplug protein transport inhibitor. Percentage of IL-12 and IL-6-producing DCs indicated, as determined by intracellular staining. Representative plots from one of three independent experiments.

Because we predict that DC precursors in the intestines are exposed to high concentrations of indole during differentiation and development, we investigated the effect of indole during BMDC generation. BMDC exposed to indole from the third day of their 7 day culture also exhibited a statistically significant inhibition of pro-inflammatory cytokine production upon LPS stimulation (Figure 9: 30% reduction in TNF and 40% reduction in IL-12, $p < 0.05$). To confirm the relevance of our observations in primary cells, we investigated indole conditioning in splenic DCs *ex vivo*. Because indole is undetectable by mass spectrometry in peripheral tissues outside the gut (Arul Jayaraman, personal communication, March 2016), it is likely that splenic DCs are indole-naïve. We treated splenocytes isolated from C57BL6/J mice with indole overnight, followed by stimulation with LPS or heat-killed Salmonella typhimurium. Production of pro-inflammatory cytokines in CD11c+ DCs was assessed via flow cytometry. We found a robust inhibition of 2-fold or greater magnitude in production of the cytokines TNF, IL-6, and IL-12 by indole-conditioned *ex vivo* splenic DCs (Figure 10). This confirmed our in vitro observations that indole inhibits pro-inflammatory cytokine production in DCs.

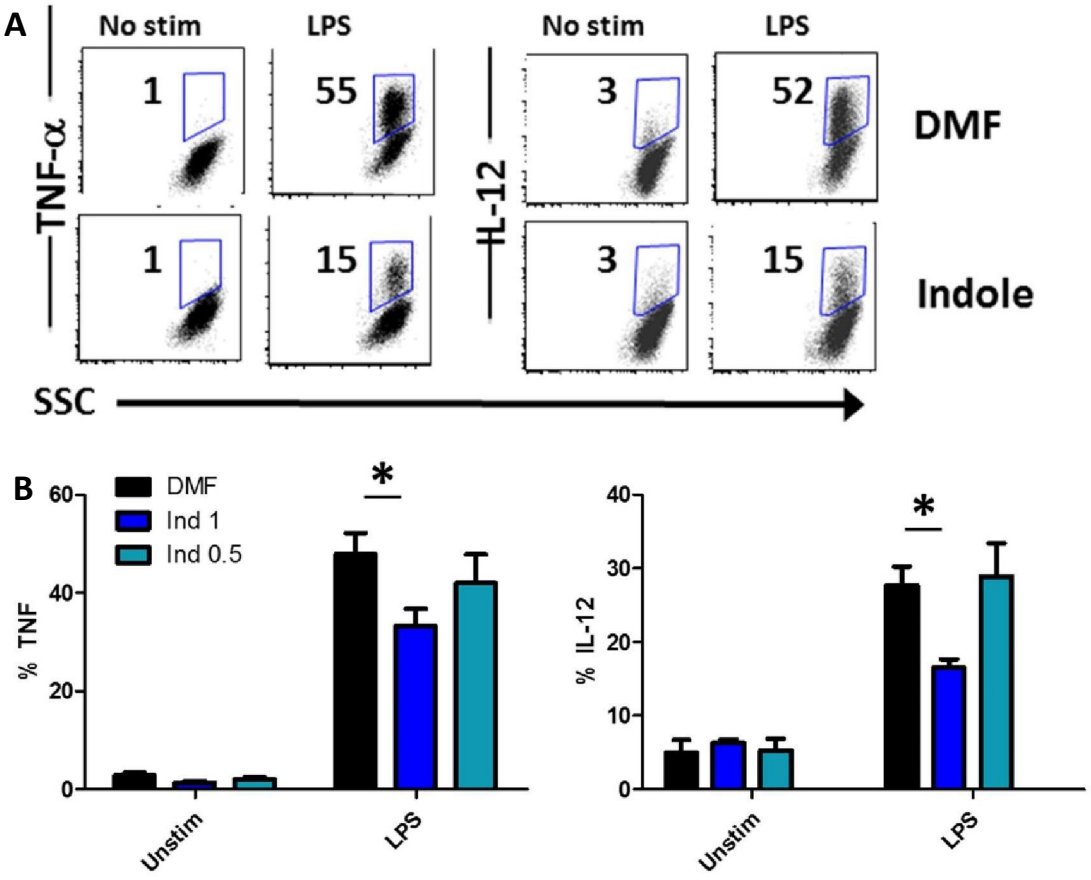

Figure 9. Conditioning with indole during differentiation inhibits TNF and IL-12 production in BMDCs.

Flow cytometric analysis of BMDCs differentiated in the presence of indole from day 3 and then stimulated with LPS (1 ug/ml) for 4 hours in the presence of Golgiplug protein transport inhibitor. Intracellular staining was performed to assess TNF and IL-12 production. (A) Representative dot plots with percentage of cytokine-producing DCs indicated. (B) Mean of three independent experiments. * $p < 0.05$ by Student's t test comparing indole-treated DCs to solvent control (DMF).

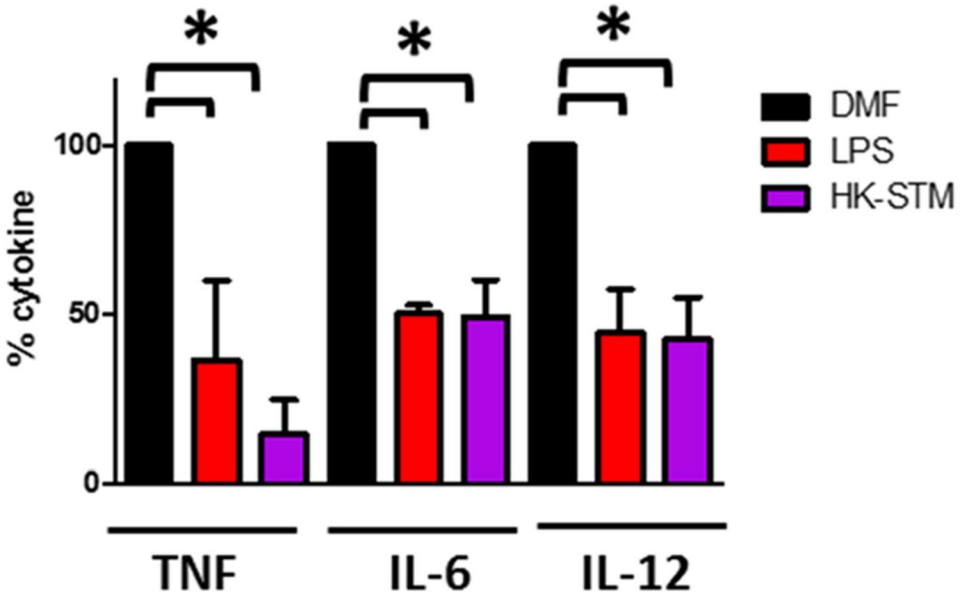

Figure 10. Indole conditioning inhibits pro-inflammatory cytokine production in splenic DCs *ex vivo*.

Flow cytometric analysis of cytokine production by indole-conditioned splenic DCs. Splenocytes were cultured for 24 hours with indole, then stimulated with LPS (5 ug/ml) or HK-STM (4 x 107 CFU) for 6 hours in the presence of Golgiplug protein transport inhibitor. Cultures were then stained for CD11c and intracellular TNF, IL-6, and IL-12. Analysis was confined to CD11c+ DCs and maximal cytokine production was normalized to solvent control (DMF). Mean of three independent experiments. * $p <$ 0.05 by Student's t test comparing indole-treated DCs to solvent control (DMF).

NF-kB

We have previously demonstrated a profound inhibition of TNF-induced NF-kB signaling in indole-treated intestinal epithelial cells (74). The ubiquitous transcription factor NF-kB is a predominant upstream effector mediating numerous inflammatory signaling pathways (95), thus we predicted that indole likely affects this signaling pathway. Using a DC2.4 cell line transduced with a dual NF-kB reporter plasmid and a GLuc reporter, we assayed NF-kB activity subsequent to overnight treatment with indole. Many ligands can activate NF-kB including growth factors, cytokines, and certain viral and bacterial products. To maintain consistency with our cytokine studies, we utilized the bacterial surface molecule LPS to induce NF-kB activity. Indeed, NF-kB signaling was inhibited in indole-conditioned cells by approximately one-third (Figure 11). This level of suppression was greater than that achieved with the positive control, TGF-β, and similar to concurrent treatment with both indole and TGF-β. The nuclear factor NF-kB pathway lies upstream and mediates transcription of many pro-inflammatory genes, including cytokines. Thus, the observed inhibitory effects of indole on pro-inflammatory cytokine production might be mediated at the level of NF-kB signaling.

To further investigate indole's effects on NF-kB signaling, we used flow cytometry to analyze the phosphorylation of p65 (RelA), a prominent subunit of the NF-kB transcription complex important for inflammatory responses (96). Here, we did not observe an effect of indole on total p65 phosphorylation or signal intensity in indole-

conditioned BMDCs stimulated with LPS (Figure 12). While this finding would seem contradictory, we have identified an alternative possibility. Our collaborator has identified that indole inhibits nuclear translocation of the NF-kB transcriptional complex in lymphatic endothelial cells (Sanjukta Chakraborty and Mariappan Muthuchamy, personal communication, August 2015). If this is also true in DCs, it would explain the inhibition of NF-kB activity despite unaffected p65 phosphorylation. Future studies will address this question by utilizing fluorescence microscopy to visualize intracellular localization of NF-kB constituents.

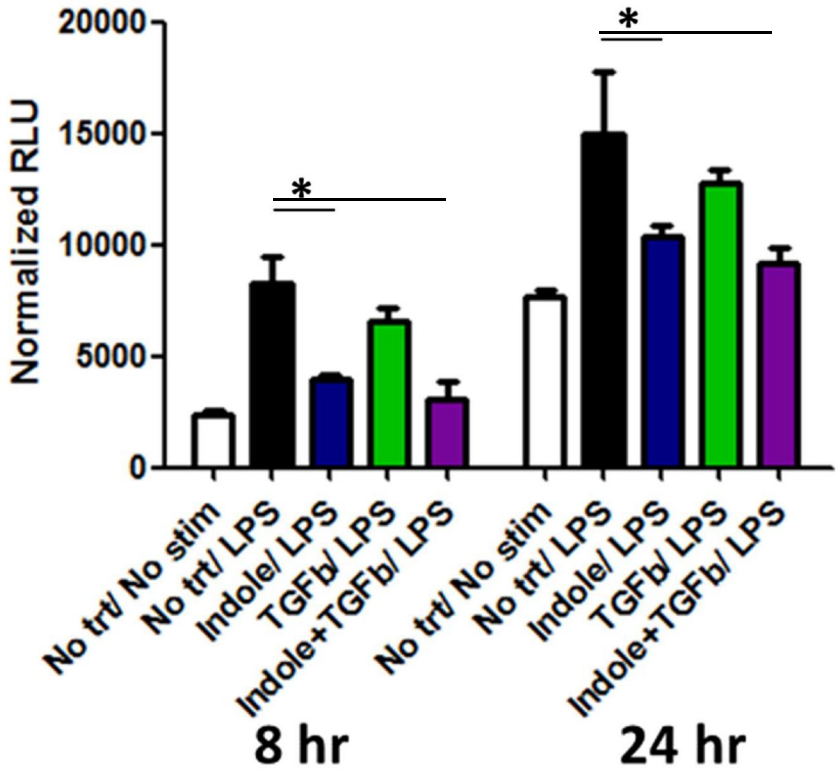

Figure 11. Indole inhibits NF-kB activity in DC2.4 cell line.

DC2.4-NFkB-GLuc cells were treated with indole +/- TGF-β for 24 hours followed by LPS stimulation (10 ug/ml) for 8 or 24 hours. Supernatants were collected and assayed for GLuc concentration in terms of relative luminescence units (RLU). * $p < 0.05$ by Student's t test comparing treated samples to untreated, LPS-stimulated sample.

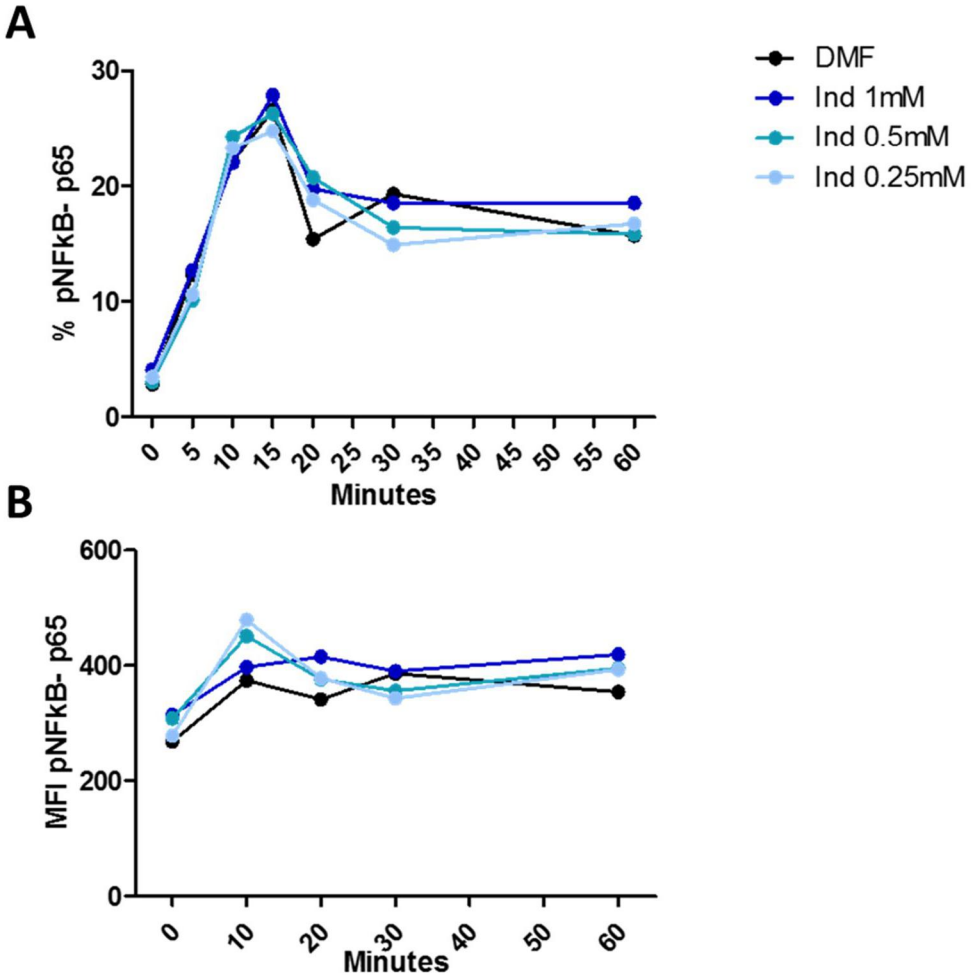

Figure 12. Indole does not alter phosphorylation of NF-kB subunit p65 in DCs.

Flow cytometric analysis of DC2.4 cells treated with indole or DMF control overnight, followed by LPS stimulation (10 ug/ml) for the indicated periods of time. Cells were stained for the phosphorylated form of the p65 NF-kB subunit and assessed by flow cytometry. (A) Percentage of DCs staining positive for p65 phosphorylation and (B) mean fluorescence intensities (MFI) of phospho-p65-positive population. One representative experiment of three shown.

Stat3

Based on our accumulating evidence that indole exerts a suppressive effect on inflammatory signaling in DCs, we decided to investigate the potential effect of indole on host-derived pro-inflammatory signals via the Signal Transducer and Activator of Transcription 3 (Stat3) pathway. To date, seven Stat proteins have been identified, having dual roles as signaling proteins in the cytoplasm as well as nuclear transcription factors. The Stat family activates a diverse set of genes including those involved with growth, survival, and inflammatory processes. The biological importance of Stat3 is evidenced by the observation that it is the only Stat whose deficiency results in embryonic lethality (97). Persistent activation of Stats occurs frequently when an upstream receptor-associated Janus kinase (JAK) becomes overactive due to genetic or epigenetic factors (98). It is common for Stat3 to be overexpressed in a variety of human cancers (99), and a role for Stat3 hyperactivity in driving DCs towards a cancerous phenotype has been established (100). In addition, Stat3 is a necessary transcription factor for pro-inflammatory Th17 differentiation. We have observed that indole inhibits the Th17 lineage (Steinmeyer et al, in preparation), thus a reasonable hypothesis would be that suppression of Stat3 signaling by indole is a mechanism by which Th17 differentiation is suppressed.

We conditioned BMDCs with indole from day 3 and then induced Stat3 signaling by stimulation with IL-6 (Figure 13). While Stat3 has both a tyrosine (Y705) and serine (S727) activation site, tyrosine phosphorylation is mediated by Janus kinases and

required for Stat3 nuclear localization and subsequent DNA binding (101), thus we queried this phosphorylation site for our study. In the absence of an external stimulus, phosphorylation of Stat3 was nearly undetectable across all conditions. Stat3 signaling was induced by the addition of IL-6, and substantial Stat3 phosphorylation (~18%) was observed in control DCs within 5 minutes of stimulation, lasting at least 60 minutes post-stimulation (peak of ~30% phosphorylated Stat3). The physiological concentration of 1 mM indole strongly inhibited Stat3 phosphorylation, with indole-treated DCs reaching peak phosphorylation of 8% at 15 minutes post-stimulation. Inhibition of Stat3 was observed not only in the percentage of DCs bearing phosphorylated Stat3 but also in the magnitude of phosphorylation on a per cell basis. The MFI values were similar between indole-treated DCs and controls in the absence of stimulation, but a 2-fold or greater suppression was observed in indole-DCs throughout the 60 minute timecourse under IL-6 stimulation. This finding is in agreement with our observation that indole inhibits the Th17 lineage and suggests that indole may inhibit Stat3 signaling across multiple immune cell types.

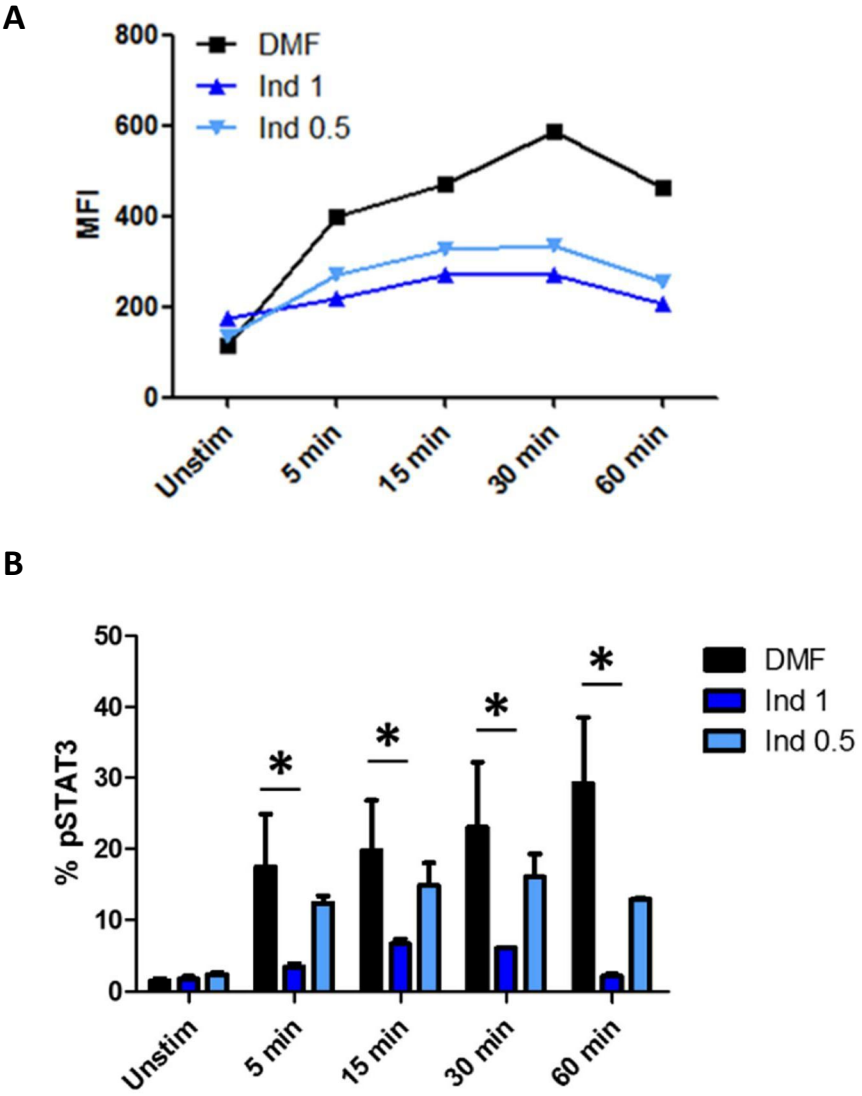

Figure 13. Indole inhibits IL-6-induced pStat3 signaling in BMDCs.

Flow cytometric analysis of BMDCs conditioned with indole from day 3 of culture and subsequently stimulated with IL-6 (10 ng/ml) for the given times. Cells were stained for phospho-Stat3 and analyzed by flow cytometry. (A) Representative MFIs from one experiment of three. (B) Mean percentage of phospho-Stat3-positive DCs from four independent experiments. * $p < 0.05$ by Student's t test comparing indole-treated DCs to solvent control (DMF) at indicated timepoints.

An important consideration is that we exclusively used IL-6 for Stat3 activation, however many other ligands can induce Stat3 including IL-10, epidermal growth factor, oncogenes, and interferons (102). An interesting subsequent study would be to assess indole's effects on Stat3 activation when stimulated by a panel of different factors. Based on our hypothesis that indole is a promotor of intestinal tolerance, one intriguing possibility would be that indole might be capable of discriminating between pro-inflammatory (ie, IL-6) and anti-inflammatory (ie, IL-10)-mediated Stat3 activation. Further work would be necessary to test this hypothesis.

Akt

Another central mediator of cellular signaling is the serine/threonine kinase, Akt (also referred to as protein kinase B), which regulates cellular processes including metabolism, growth, proliferation, and survival. The Akt pathway is activated by numerous receptor tyrosine kinases, cytokine receptors, G-protein-coupled receptors, and other molecules which activate the lipid kinase PI3K and result in production of phosphatidylinositol (3,4,5)-trisphosphate (PIP3). This second messenger acts as a docking site at the plasma membrane for Akt where phosphorylation and activation occurs. Akt is located upstream of NF-kB and can induce NF-kB signaling via phosphorylation of IKKα and Tpl2 (103). We chose to interrogate the effect of indole on Akt phosphorylation to determine whether indole's inhibition of NF-kB might be a secondary effect due to modulation of an upstream target. Our investigation revealed that treatment with 1 mM indole inhibited LPS-induced Akt phosphorylation in DCs (Figure

14). This difference was observed at 30 minutes post-LPS stimulation, where phospho-Akt was reduced by over 50% with indole pre-treatment. No difference was observed between indole-DCs and controls in the absence of external stimulation, which indicates that basal Akt signaling is unaffected by indole. Instead, an inflammatory insult appears necessary for indole to dampen Akt phosphorylation. This is important as Akt is ubiquitously expressed in DCs and mediates many essential cellular processes, so a complete ablation of Akt signaling would be detrimental. Whether downstream inhibition of NF-kB is a direct result of indole's effects on Akt or mediated through a distinct pathway remains to be determined. Either way, a robust inhibitory effect of indole on multiple pro-inflammatory signaling pathways in DCs has been revealed.

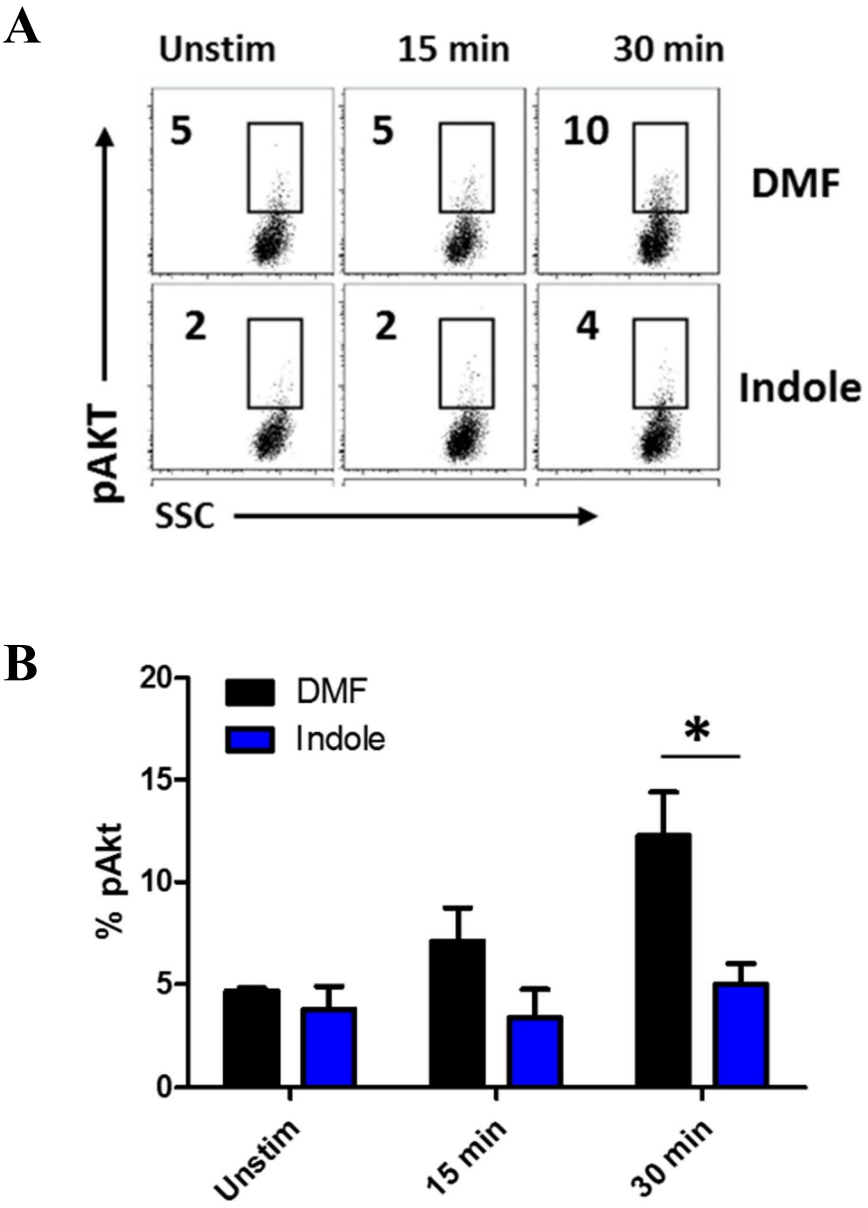

Figure 14. Indole inhibits pAkt signaling in BMDCs.

Flow cytometric analysis of BMDCs conditioned with indole from day 3 of culture and subsequently stimulated with LPS (5 ug/ml) for the given times. Cells were stained for phospho-Akt and analyzed by flow cytometry. (A) Representative plots with numbers indicating the percentage of pAkt-positive DCs. (B) Mean percentage of phospho-Akt-positive DCs from three independent experiments. * $p < 0.05$ by Student's *t* test comparing indole-treated DCs to solvent control (DMF) at indicated timepoints.

Anti-inflammatory cytokines

Based on the close proximity of mucosal DCs to indole in the lower GI tract, we predicted that indole may affect specific mucosal DC properties. A well-known regulator of immune function abundantly present in the GI tract is TGF-β. This cytokine possesses potent anti-inflammatory properties, evidenced by TGF-β-null mice undergoing severe inflammation, autoimmunity, and premature death within 2 weeks of birth (104). When added to our system, a marked additive effect upon concurrent indole and TGF-β treatment was observed in DCs (Figure 15). This finding is striking as it demonstrates synergistic effect of two highly-abundant intestinal metabolites, one host-derived and one microbiota-derived.

To determine whether this was a specific downregulation of pro-inflammatory cytokines, we also examined production of the canonical DC-produced anti-inflammatory cytokines, IL-10 and TGF-β. Neither IL-10 nor TGF-β production were affected by indole conditioning (Figure 16). Overall these results indicate that indole selectively suppresses pro-inflammatory cytokines and signaling in DCs, resulting in a net anti-inflammatory effect.

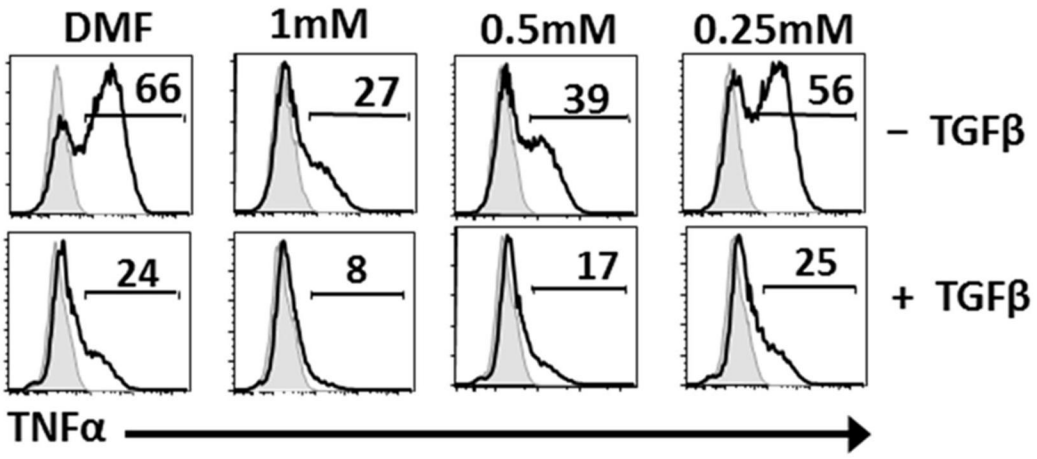

Figure 15. Indole synergizes with TGF-β to inhibit TNF production in DCs.

Flow cytometric analysis of DC2.4 cells treated with indole or DMF control for 24 hours in the presence or absence of TGF-β (10 ng/ml). DCs were then stimulated with LPS (1 ug/ml) for 4 hours in the presence of Golgiplug protein transport inhibitor and stained for intracellular TNF. Shaded gray areas represent unstimulated samples. Representative histograms from one of three independent experiments.

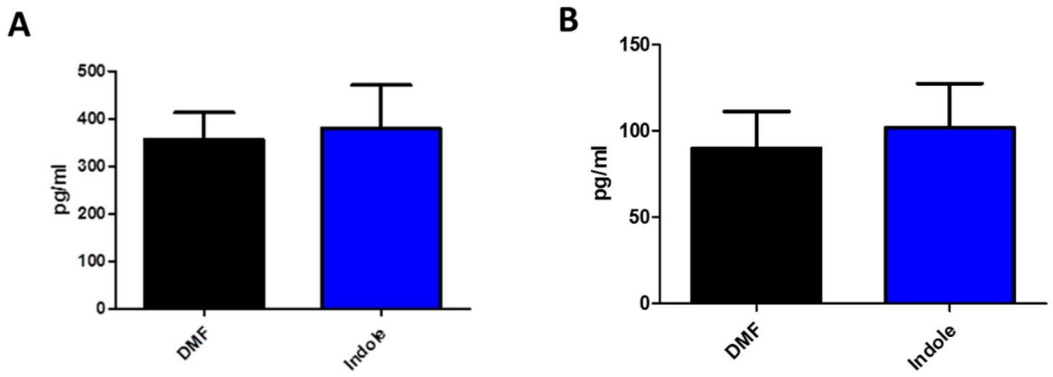

Figure 16. Indole does not affect IL-10 or TGF-β production in DCs.

BMDCs were cultured from day 3 with indole (1 mM) or DMF solvent control. On day 7, DCs were washed, counted, and plated with LPS (1 ug/ml) for 24 hours. Supernatants were collected and analyzed for cytokines (A) IL-10 and (B) TGF-β using commercially available ELISA kits. No significant differences were observed between indole and DMF-treated DCs in four replicate experiments. ($p < 0.05$ by Student's t test)

Aryl hydrocarbon receptor (AhR)

Thus far, we have revealed novel biology involving the direct effect of indole on DC signaling and cytokine production. Our previous identification of indole as an AhR ligand (83) led us to the prediction that the observed effects of indole on DCs might be mediated through this pathway. In support of this hypothesis, others have reported that activation of the AhR pathway by treatment with the canonical AhR ligand TCDD lessened pro-inflammatory signs of colitis in a murine DSS model (105).

To evaluate the possible role of AhR in mediating the anti-inflammatory effects of indole on DCs, we acquired AhR-deficient mice and analyzed splenic and bone marrow-derived DCs. We first treated splenocytes from AhR-deficient and WT mice overnight with indole, followed by stimulation with heat-killed S. typhimurium. Consistent with earlier reports, we observed an overall increase in TNF production (~ 40%) by TLR-stimulated APCs from AhR-deficient mice (106). Nonetheless, a 2-fold inhibition of TNF production was observed in indole-treated DCs derived from both WT and AhR-/- mice, indicating that the effect was independent of AhR (Figure 17). Similarly, we observed nearly equivalent inhibition of phospho-signaling in indole-DCs generated from WT or AhR-/- bone marrow in both our Stat3 (Figure 18) and Akt (Figure 19) experiments. Collectively, these experiments establish that indole's inhibition of pro-inflammatory signaling in DCs is entirely independent of AhR.

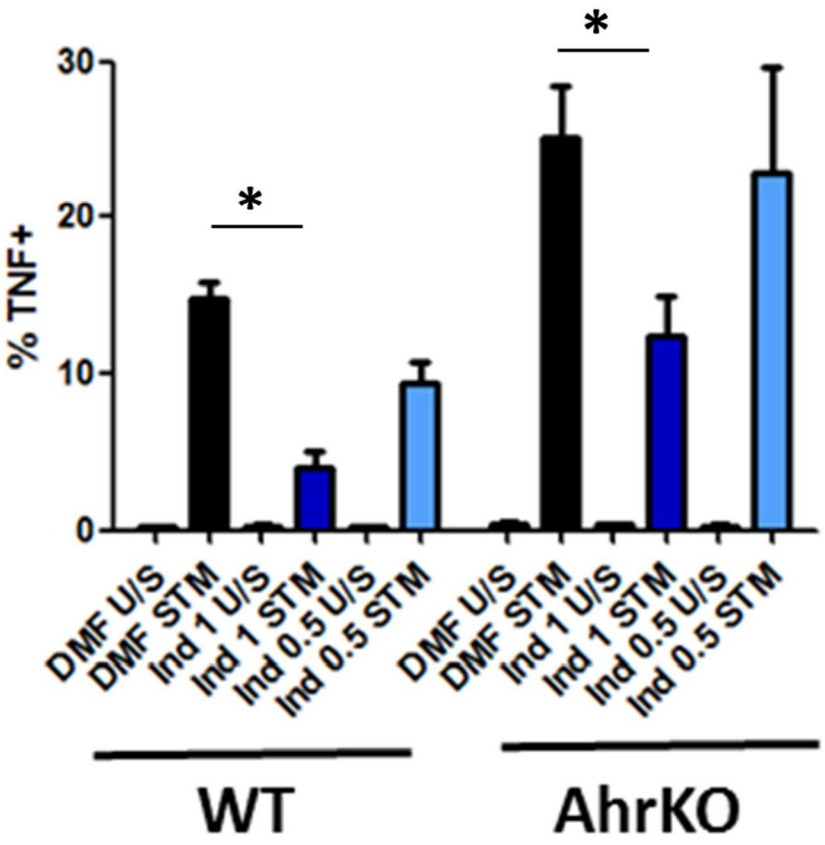

Figure 17. The indole-mediated inhibition of TNF production in DCs is AhR-independent.

Splenocytes from WT or AhrKO mice were treated with indole or DMF overnight, followed by stimulation for 6 hours with HK-STM (4 x 107 CFU) in the presence of Golgiplug protein transport inhibitor. Cells were surface stained for CD11c to distinguish DCs followed by intracellular staining for TNF production. Flow cytometric analysis was confined to CD11c+ cells. * $p < 0.05$ by Student's *t* test comparing indole-treated DCs to solvent control (DMF).

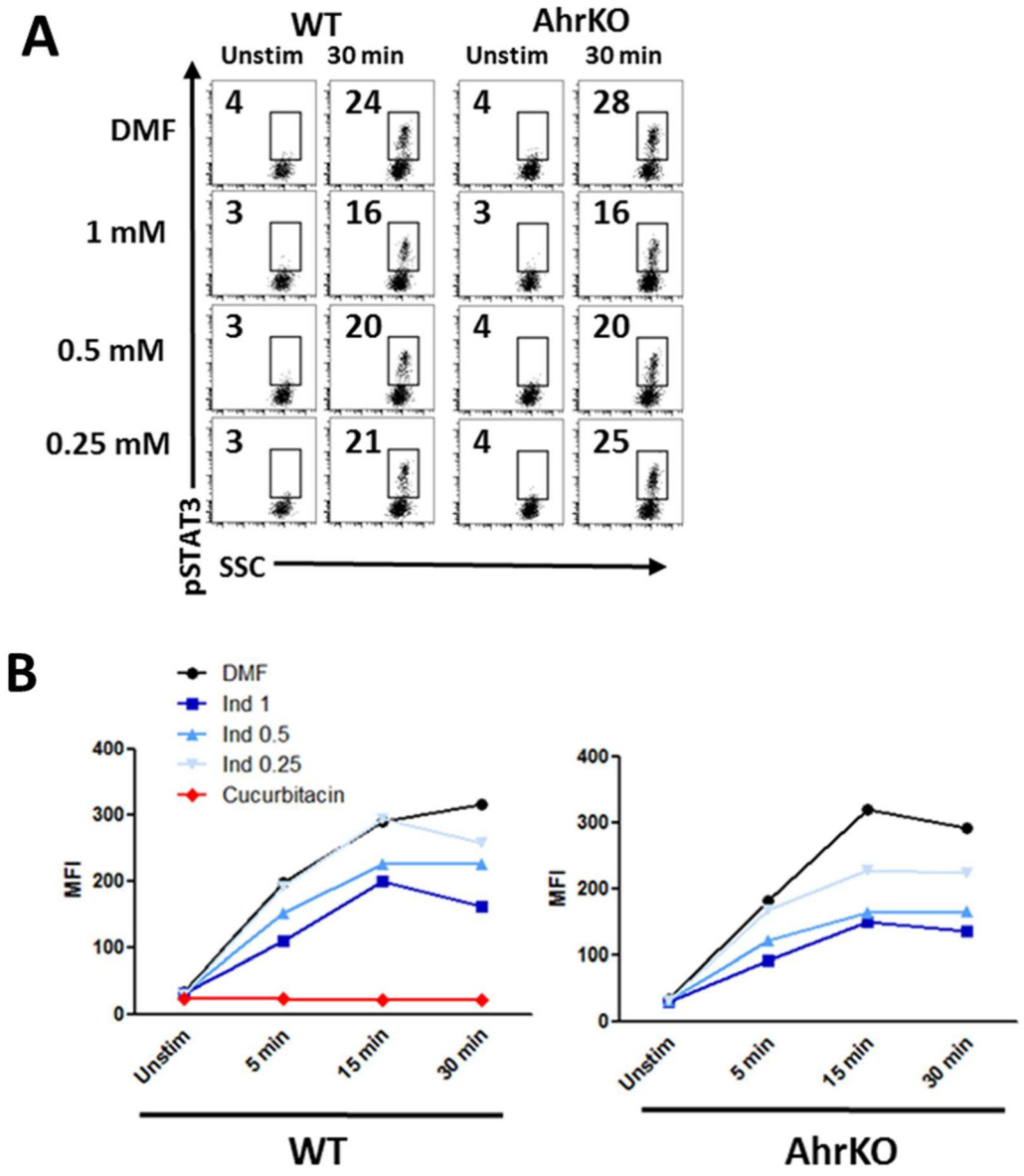

Figure 18. The indole-mediated inhibition of pStat3 signaling in DCs is AhR-independent.

Flow cytometric analysis of BMDCs from WT or AhrKO mice cultured in the presence of indole or DMF from day 3. DCs were stimulated with IL-6 (10 ng/ml) for the given times and stained for phospho-Stat3, which was detected by flow cytometry. Stat3 signaling was reported as (A) percent of phosphorylated cells and (B) MFI of the Stat3 positive population. The specific Stat3 inhibitor, cucurbitacin I, was included as a negative control. Representative data from one of four independent experiments.

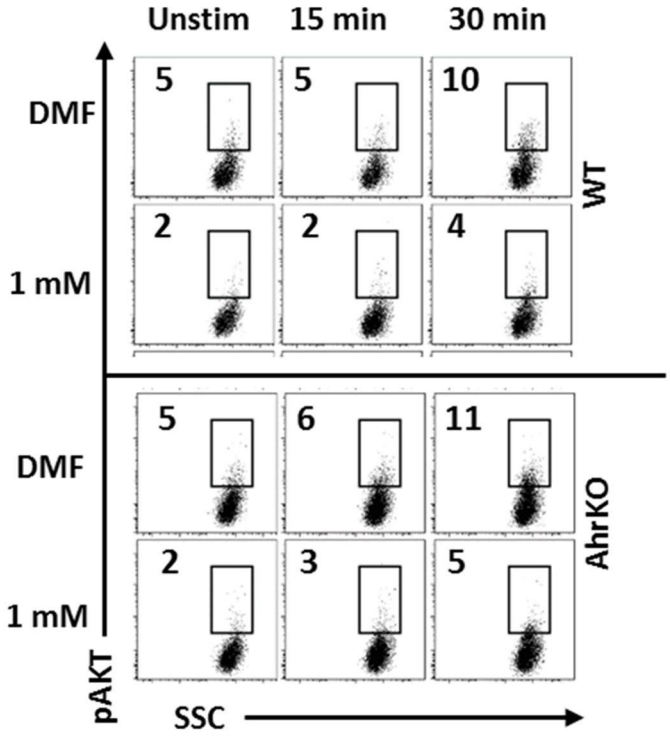

Figure 19. The indole-mediated inhibition of pAkt signaling in DCs is AhR-independent.

Flow cytometric analysis of BMDCs from WT or AhrKO mice cultured in the presence of indole (1 mM) from day 3. DCs were stimulated with LPS (5 ug/ml) for the given times and stained for phospho-Akt, which was detected by flow cytometry. Percentage of pAkt-positive DCs indicated. Representative data from one of three independent experiments.

Indole does not alter antigen-presenting cell properties of dendritic cells

To ensure that our observations were not due to indole-induced alteration of the inherent functional capacity of DCs, we performed a series of assays looking at the general functions of DCs. We first treated DCs with indole and assessed their viability by measuring lactate dehydrogenase release as well as propidium iodide uptake. Results from both assays indicated a low level of cellular death which did not differ from solvent controls, confirming that indole did not induce cytotoxicity in DCs (Figure 20). The ability of antigen-presenting cells to respond to bacterial LPS is dependent upon the expression of TLR4 on their surface. We compared TLR4 expression level in DC2.4 and BMDC treated for 18 hours with indole as well as BMDC cultured with indole from day 3 and found that indole does not alter surface TLR4 expression on DCs (Figure 21). In the periphery, DCs exist in an immature state with a high phagocytic capacity. Upon encountering and engulfing antigen, DCs upregulate their expression of maturation markers including MHC-II and the co-stimulatory molecule, CD86. This stimulation triggers the migration of DCs to local lymphoid organs and prepares them for presentation of peptide fragments to naïve T cells. We treated BMDC with indole from day 0, 3, or 6 in culture prior to LPS stimulation. Flow cytometric analysis of MHC-II and CD86 expression revealed that the presence of indole did not alter DC maturation compared to solvent controls (Figure 22).

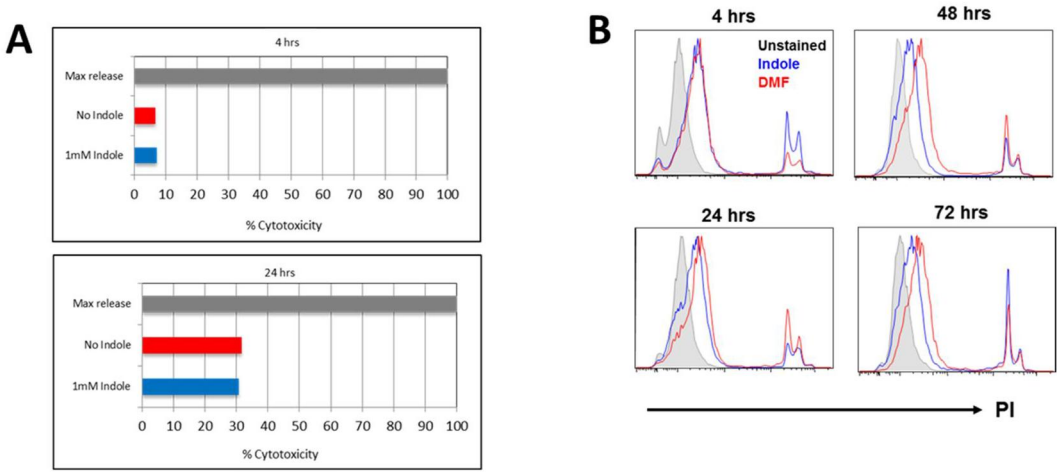

Figure 20. Indole does not alter DC viability.

DC2.4 cells were cultured with or without indole (1 mM) for the indicated timepoints and cell viability was assessed. (A) Percent cytotoxicity was calculated as a measure of the cytosolic enzyme lactate dehydrogenase. (B) Propidium Iodide (PI) labeling was performed at the indicated timepoints and percent viability was determined by flow cytometry. Representative data from one of three independent experiments.

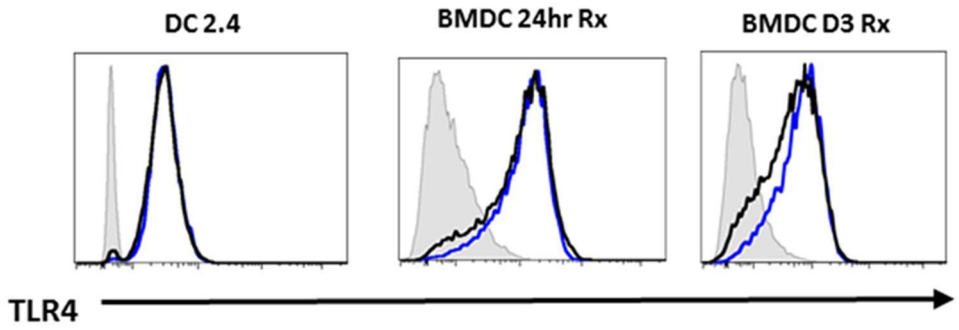

Figure 21. Indole does not alter TLR4 expression in DCs.

Flow cytometric analysis of surface TLR4 expression following treatment with indole (1 mM) for 24 hours (DC2.4 and BMDC) or during development from day 3 (BMDC D3). Blue line represents indole-treated DCs, black line represents DMF solvent control, and gray shaded areas are unstained controls. Representative data from one of three independent experiments.

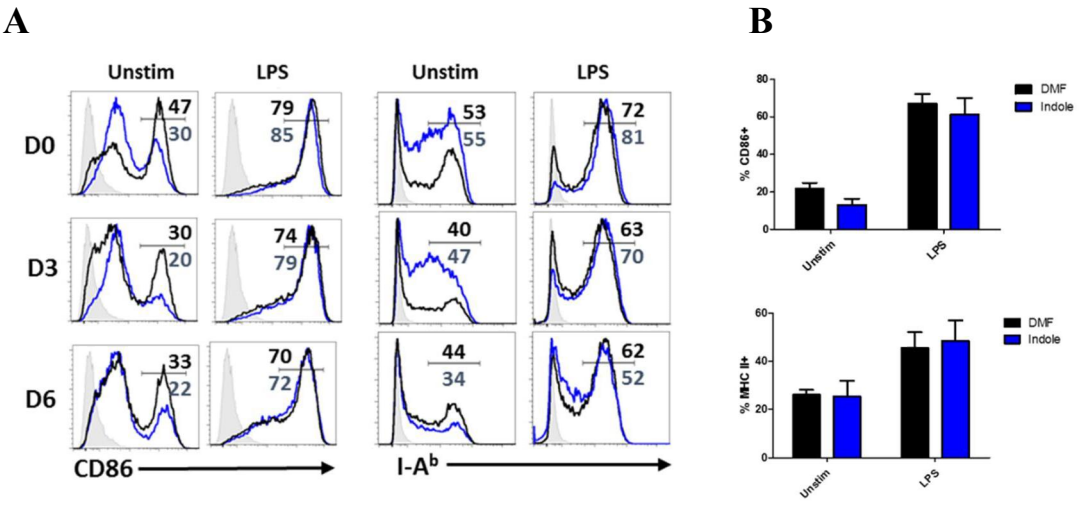

Figure 22. Indole does not alter DC maturation.

Flow cytometric analysis of BMDCs treated with indole (1 mM) from day 0, 3, or 6 of differentiation, then cultured for 24 hours in the presence or absence of LPS (1 ug/ml) and surface stained for CD86 and MHC-II (I-Ab). (A) Representative histograms where blue line represents indole-treated DCs, black line represents DMF controls, and shaded gray areas are unstained controls. Mean fluorescent intensity (MFI) indicated on representative FACS plots. (B) Mean percentage of DCs expressing surface CD86 (upper panel) or MHC-II (lower panel). No significant differences observed between indole- treated DCs and DMF controls in four replicate experiments. ($p < 0.05$ by Student's *t* test)

Production of reactive oxygen species (ROS) by DCs is an established component of DC-T cell interactions (107, 108). We investigated the secretion of reactive oxygen species (ROS) and nitric oxide (NO) in DC2.4 and BMDC. Our results confirmed that these properties of DCs were also intact regardless of indole treatment (Figures 23 and 24). The phagocytic ability of DCs was examined by incubating DCs with FITC-labeled OVA particles, then analyzing via flow cytometry. Indole did not alter particle uptake by DCs (Figure 25). Together, these results indicate that indole does not interfere with general APC functions of DCs but is instead selectively dampening inflammation through some alternative mechanism.

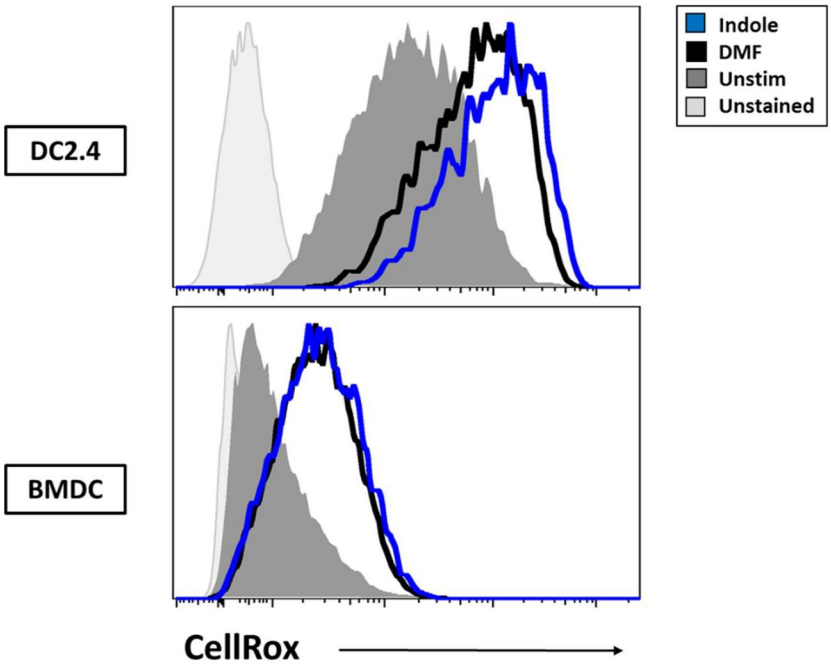

Figure 23. Indole does not alter reactive oxygen species production in DCs.

Flow cytometric analysis of DC2.4 (upper panel) and BMDCs (lower panel) cultured in the presence of indole (1 mM) or DMF for 18 hours, then labeled with CellRox and stimulated with LPS. The presence of ROS oxidizes CellRox reagent to a fluorescent state, which was subsequently analyzed via flow cytometry. Representative data from one of three independent experiments.

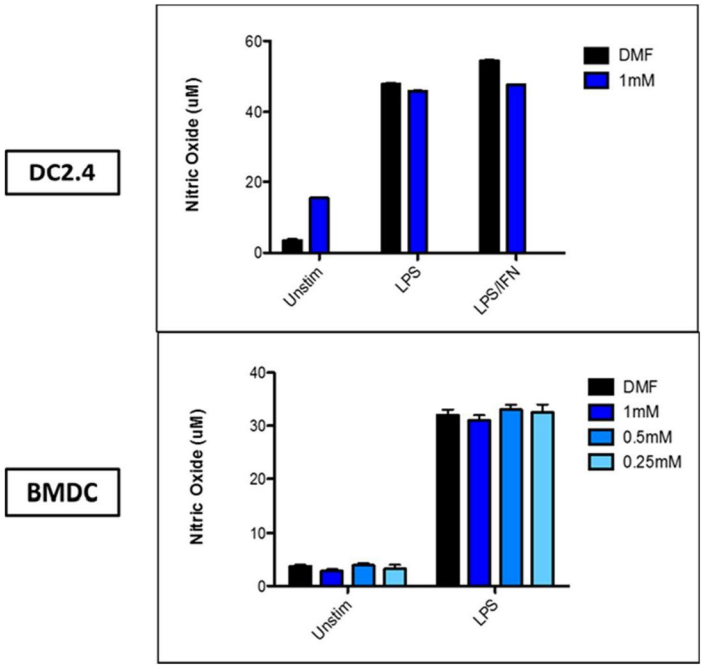

Figure 24. Indole does not alter nitric oxide production in DCs.

DC2.4 cells (upper panel) or BMDCs (lower panel) were cultured with indole or DMF for 24 hours in the presence of either LPS, LPS and IFNγ, or no stimulation. Nitric oxide (NO) content in the supernatant was measured by the Griess reaction. No significant differences in NO production observed between indole-treated DCs and DMF controls in three replicate experiments. ($p < 0.05$ by Student's *t* test)

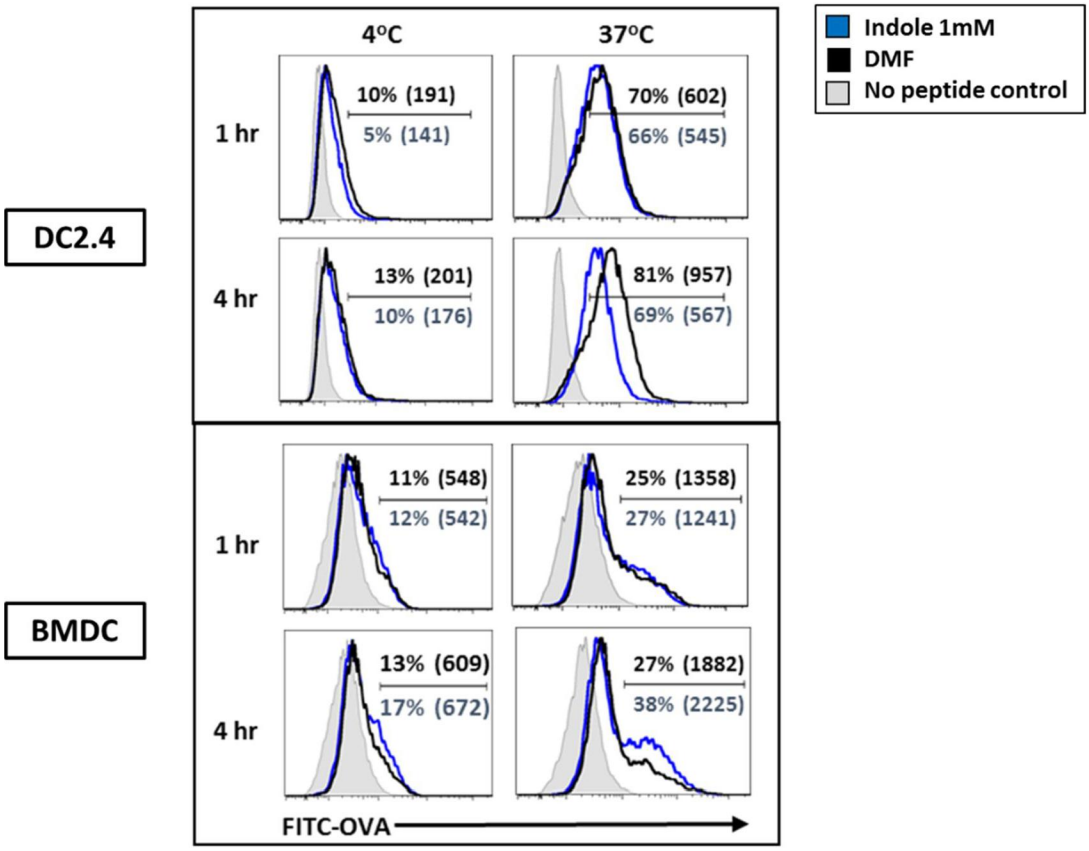

Figure 25. Indole does not alter antigen uptake in DCs.

Flow cytometric analysis of antigen uptake by DC2.4 (upper panel) and BMDCs (lower panel). Subsequent to 24 hour indole conditioning, DCs were incubated with Ovalbumin (OVA)-FITC conjugate at 4°C or 37°C for 1 or 4 hours. BMDCs were surface stained for CD11c and all DCs were fixed with paraformaldehyde. Uptake of FITC-OVA antigen was measured by flow cytometry. Percentage and MFI of FITC-OVA-positive DCs indicated on histograms. Representative data from one of three independent experiments.

Indole induces homing markers on dendritic cells

Dendritic cell subsets bear numerous surface antigens that regulate their migration and localization. Because indole is produced in the gut and appears to induce anti-inflammatory properties in DCs, we hypothesized that indole might affect migration and retention in mucosal tissues. Thus, we looked at expression of the gut homing marker, chemokine receptor CCR9. Remarkably, we observed that indole strongly induced CCR9 on BMDC, indicating that indole imprints DCs with gut-homing specificity (Figure 26). Retinoic acid was included as a positive control. RA is present in murine plasma at approximately 1 uM (109) and is predicted to exist at higher concentrations in tissue microenvironments *in vivo* (110), thus we chose to use 1 uM as a conservative estimate of relevant physiological concentration. Indole's effect was even more profound than that of retinoic acid, a known inducer of mucosal homing markers on T cells (53). Surface expression of CCR9 on indole-conditioned DCs reached 34%, compared to 22% on RA-conditioned DCs and 10% on solvent controls. Upregulation of this gut homing marker would suggest that in addition to suppressing pro-inflammatory signaling, indole imparts gut homing signals upon DCs to preferentially maintain them within the intestinal environment.

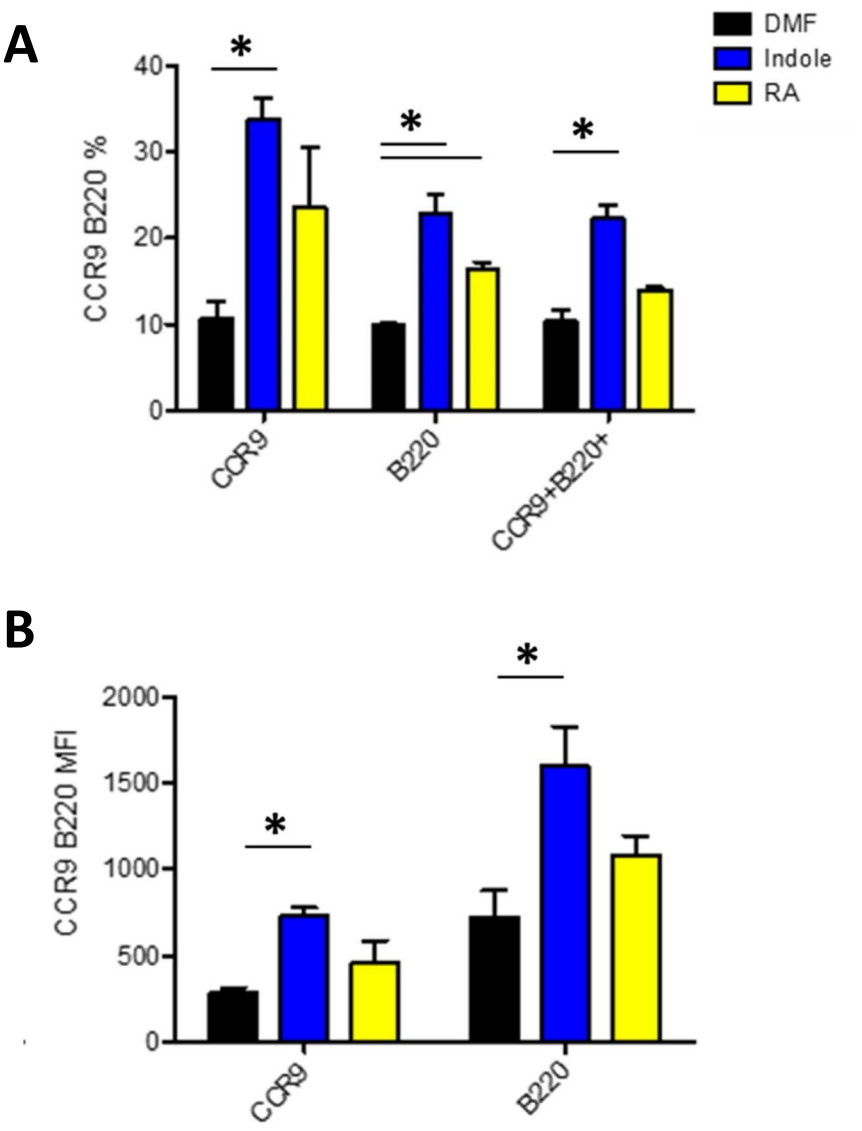

Figure 26. Indole induces CCR9 and B220 on DCs.

BMDCs were cultured in the presence of indole (1 mM), retinoic acid (1 uM), or DMF solvent control from day 3. On day 7, DCs were surface stained for expression of CCR9 and B220 and analyzed by flow cytometry. (A) Data reported as percentage of surface marker-expressing DCs and (B) corresponding MFI values. Mean of three independent experiments. * indicates $p < 0.05$ for one-way ANOVA followed by Dunnett's post-test.

One possible explanation for these observations would be that indole promotes retinoic acid availability to the DCs, thus inducing gut homing markers indirectly by providing substrate for a previously identified mechanism. To investigate this possibility, we analyzed aldehyde dehydrogenase (ALDH) activity via flow cytometry using the fluorescent ALDH substrate, Aldefluor. Surprisingly, we found that indole does not affect ALDH activity in DCs (Figure 27). We confirmed this observation by assessing gene expression of aldehyde dehydrogenase family 1 subfamily A2, a rate-limiting enzyme for retinal metabolism in DCs (Figure 27). Thus, indole utilizes a novel mechanism for inducing gut-homing markers on DCs, apparently independent of retinoic acid availability.

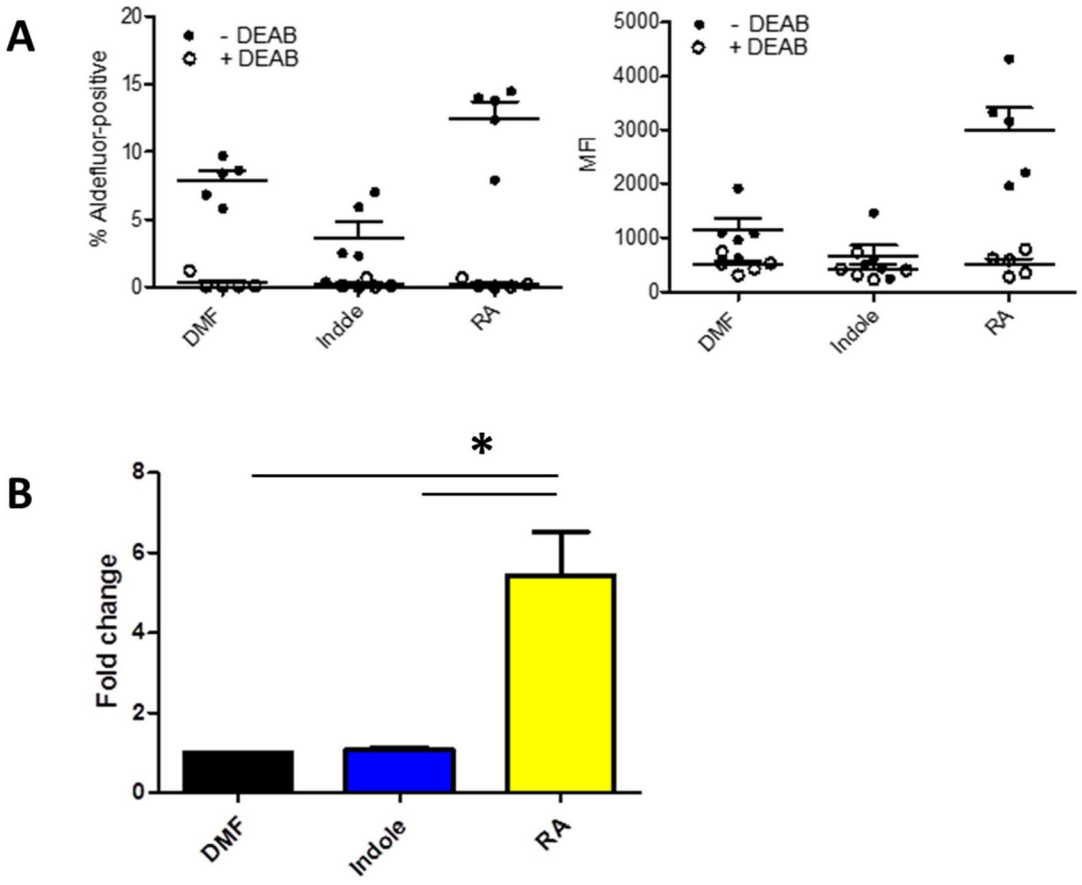

Figure 27. Indole does not induce aldehyde dehydrogenase activity in DCs.

(A) BMDCs cultured from day 3 with indole (1 mM), retinoic acid (1 uM), or DMF control were assayed for aldehyde dehydrogenase (ALDH) activity using the Aldefluor commercial kit. The ALDH inhibitor, DEAB reagent, was included to control for background fluorescence. Aldefluor fluorescence as a measure of ALDH activity was analyzed by flow cytometry. Data reported as percentage of Aldefluor positive DCs (left panel) and corresponding MFI values (right panel). Individual dots represent data from five independent experiments. (B) Gene expression of aldh1a2 was assayed by real-time qPCR in day 7 BMDCs cultured with indole from day 3. Gene expression was normalized to 18s ribosomal RNA. Mean of three independent experiments. * $p < 0.05$ by Student's *t* test.

We also observed an indole-induced upregulation of the B220 (CD45R) antigen (Figure 26), which is widely used to distinguish the plasmacytoid DC lineage and is believed to contribute to gut homing. However, more recently B220 has been reported to distinguish a DC subset more closely developmentally related to conventional rather than plasmacytoid DCs and a precursor to resident CD8+ and CD8- conventional DCs (111). This upregulation denotes a differential conditioning effect between RA and indole, as RA does not induce B220 on BMDCs (110). Further inquiry would be necessary to determine the physiological importance of indole's upregulation of B220 on BMDCs.

Encountering maturation-inducing stimuli induces an upregulation of the chemokine receptor CCR7 in DCs. This shift promotes DC migration from peripheral tissues to the lymph nodes to present captured antigen. CCR7 expression facilitates this migration by rendering DCs sensitive to the chemoattractants, CCL19 and CCL21, which direct DCs to T cell areas of lymphoid tissues (112). We observed that indole conditioning during BMDC development resulted in a pronounced increase in expression of CCR7 in day 7 freshly harvested DCs, approximately 5-fold greater than RA or solvent controls (Figure 28). Exposure to LPS is a DC-activating signal known to upregulate CCR7 expression in order to prime DCs for lymph node entry. Consistent with the literature, exposure to LPS for 24 hours further induced CCR7 expression in our BMDC cultures overall. Here, indole-conditioned DCs once again expressed a higher level of CCR7 expression compared to controls by over 3-fold in magnitude (Figure 28). This enhancement in

CCR7 expression would suggest a superior capacity for lymph node homing and entry by indole-conditioned DCs.

We utilized an in vitro chemotaxis assay to test for functional relevance of the observed CCR7 and CCR9 upregulation by indole. Consistent with the literature, DCs grown in the presence of RA exhibited enhanced migration to the CCR9 ligand, CCL25, while migration towards CCR7 ligands were unaffected (53, 113). However, indole-conditioned BMDCs did not demonstrate enhanced migration towards either ligand (Figure 29). While unexpected, it is important to consider the caveat that this assay was performed in vitro and does not accurately mimic the true physiological environment. To further this line of study, we propose the use of an *in vivo* transfer of indole-conditioned and control DCs to identify their true capacity of migration to physiological ligands at their target sites.

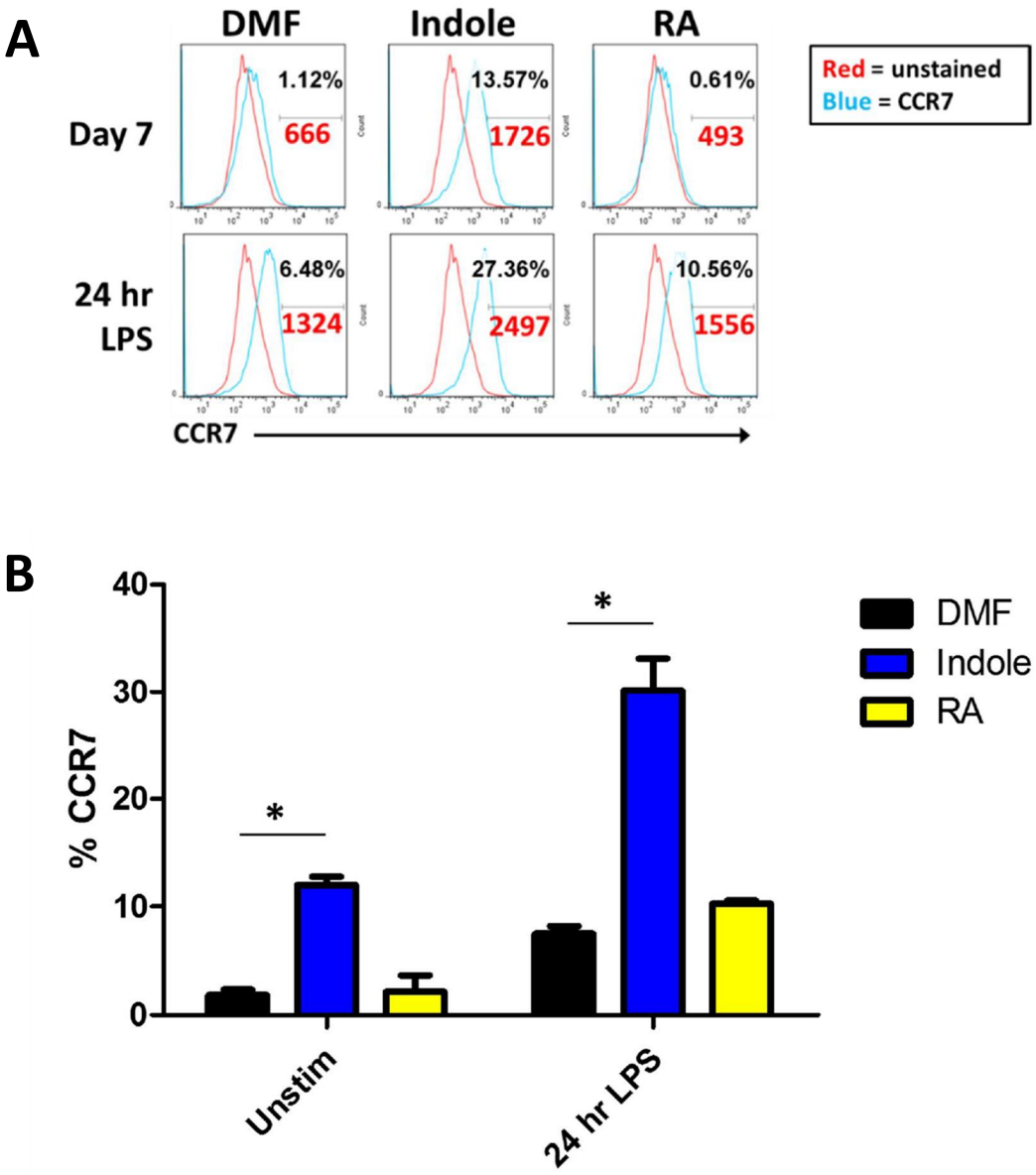

Figure 28. Indole upregulates CCR7 expression in DCs.

Flow cytometric analysis of BMDCs cultured from day 3 in the presence of indole (1 mM), RA (1 uM), or DMF control and either surface stained for CCR7 expression directly or treated with LPS (1 ug/ml) for 24 hours prior to CCR7 staining. (A) Representative histograms with percentage of CCR7-positive DCs and corresponding MFIs indicated. (B) Mean of three independent experiments. * indicates $p < 0.05$ for one-way ANOVA followed by Dunnett's post-test.

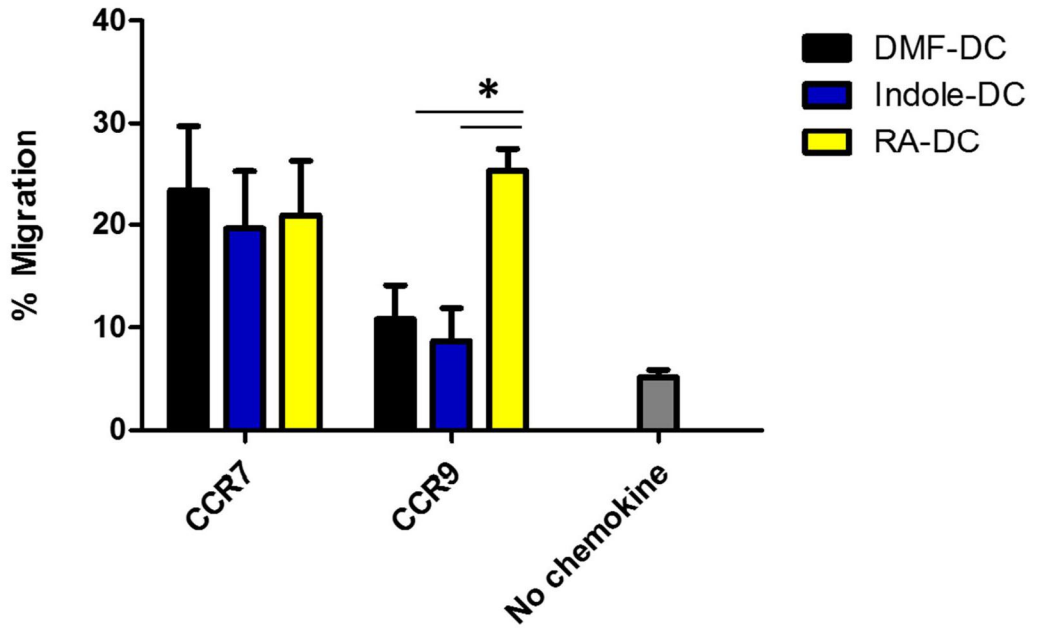

Figure 29. Indole does not enhance DC migratory capacity to CCR7 and CCR9 ligands in vitro.

Day 7 BMDCs conditioned from day 3 with indole (1 mM), RA (1 uM), or DMF control were placed in the upper chamber of a transwell cell culture plate with 8.0 uM polycarbonate membrane. Chemokines CCL19 and CCL21 (CCR7 ligands) or CCL25 (CCR9 ligand) were added to the bottom chamber at a concentration of 1 ug/ml each. Cells were incubated for 3 hours at 37°C, then migrated DCs in the bottom chamber were recovered and counted. Migration is reported as a proportion of the initial population in the upper chamber. Transwells without chemokines were included as a negative control for migration. Mean of three independent experiments. * $p < 0.05$ by Student's *t* test.

To test whether indole non-specifically upregulates all chemokine receptors, we also analyzed CCR2, the cell surface receptor for Monocyte Chemoattractant Proteins (MCPs). CCR2 recruits immune cells to sites of inflammation and tissue injury (114) and would be considered an irrelevant marker as our studies are focused on DCs of the intestinal tract. We found no appreciable difference in CCR2 expression between indole-conditioned and control DCs (Figure 30). This finding supports the prevailing paradigm that indole selectively modifies expression of certain surface antigens on DCs, including those that have a beneficial role in the gut.

The membrane-spanning integrin, CD103, is present on numerous immune cell types including intraepithelial T cells, some peripheral Tregs, and lamina propria cells. Most DCs in the small intestinal lamina propria express CD103 (115), as well as DC subsets in the Peyers Patches (116), colonic lamina propria and mesenteric lymph nodes (46). Dendritic cells bearing CD103 possess specific mucosal properties: carrying antigen from the intestines to the mesenteric lymph nodes (117, 118), inducing gut homing markers on T cells (116), and potently inducing regulatory T cells (54). In addition, DC expression of CD103 has been reported to be necessary for suppressing T cell-mediated colitis in an adoptive transfer model (119).

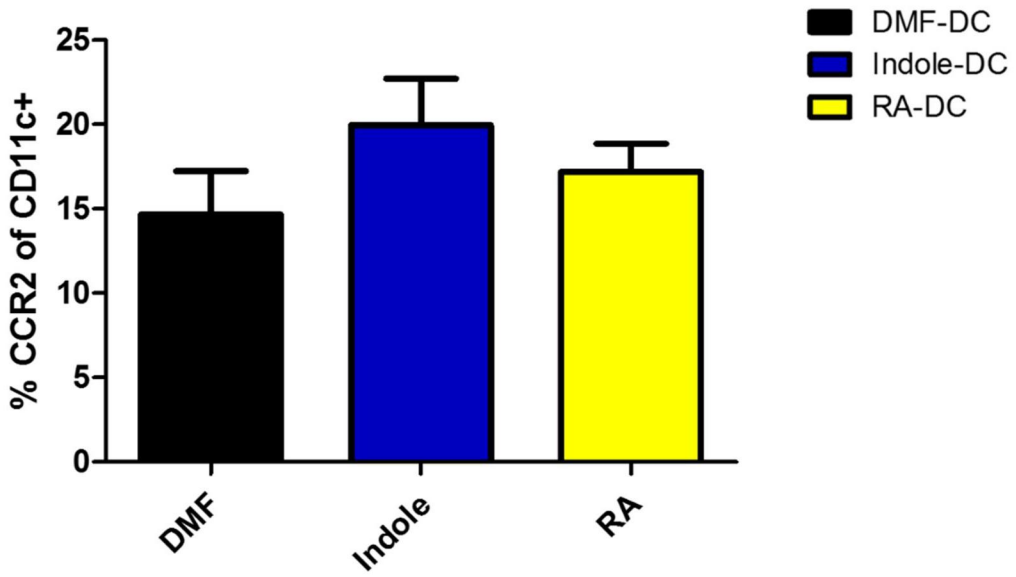

Figure 30. Indole does not affect CCR2 expression on DCs.

Day 7 BMDCs conditioned from day 3 with indole (1 mM), RA (1 uM), or DMF control were surface stained for expression of CCR2 and analyzed by flow cytometry. No significant differences in CCR2 expression observed between indole-treated DCs and DMF controls in three replicate experiments. ($p < 0.05$ by Student's *t* test)

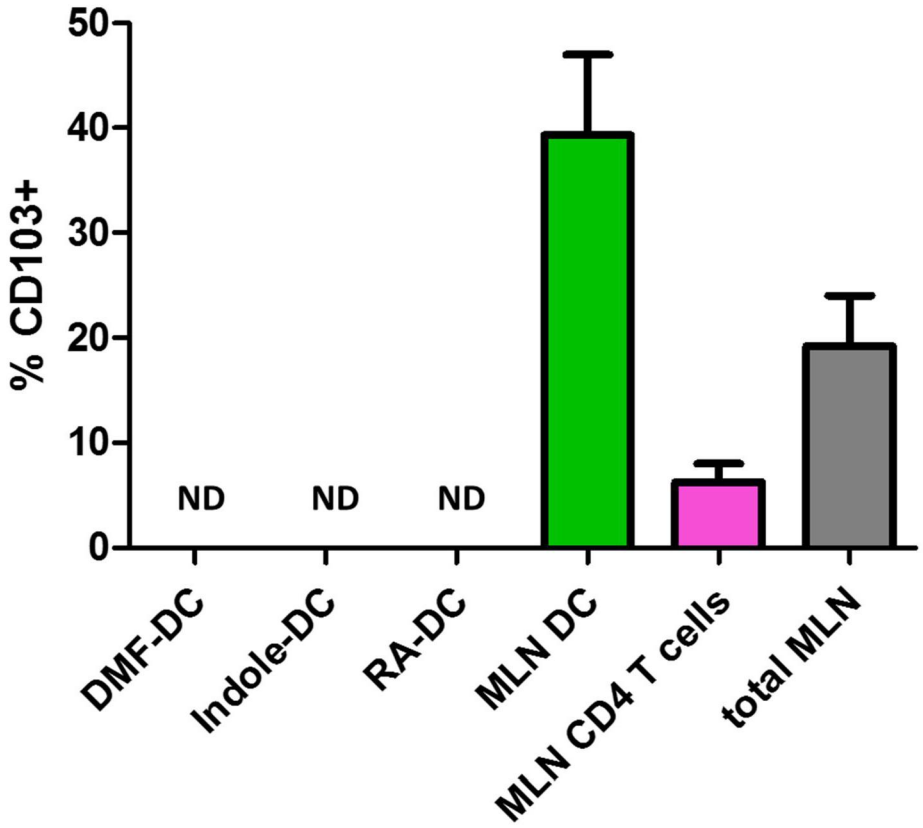

Figure 31. Indole does not induce CD103 expression in BMDCs in vitro.

BMDCs conditioned from day 3 with indole (1 mM), RA (1 uM), or DMF control were surface stained for expression of CD103 and analyzed by flow cytometry. No induction of CD103 expression observed in BMDCs under any treatment across three replicate experiments. ($p < 0.05$ by Student's *t* test) Mesenteric lymph node cells included as positive control for staining. ND, not detected.

The mechanism by which CD103 expression is regulated remains unresolved, but one possibility is that expression is driven by contact with components of the intestinal microenvironment. Thus, we sought to test whether indole induces CD103 expression on dendritic cells. Our results revealed that indole conditioning during development of bone marrow-derived DCs did not induce CD103 expression (Figure 31), which suggests that indole does not confer all mucosal properties upon DCs. However, it is important to note that while retinoic acid is an established inducer of CD103 expression on DCs *in vivo*, it is insufficient for inducing CD103 on BMDCs in vitro (110). This suggests that other factors in addition to retinoic acid are necessary for DC CD103 induction. Thus, the possibility that indole plays a partial role in mediating CD103 DC expression *in vivo* cannot be discounted.

Discussion

In this chapter, we have revealed a novel and previously unappreciated modulation of DC phenotype and function by a single endogenous microbiota metabolite. To our knowledge, the effect of indole on DCs has not been interrogated. Previous investigations by our group and others have focused on the immune modulatory effects of indole on intestinal epithelial cells and naïve T cells (74, 81). In this study, we have expanded our efforts to include dendritic cells, which play a critical role in mediating peripheral tolerance in the lower GI tract. As predicted, conditioning with indole appears to suppress subsequent pro-inflammatory responses by DCs in response to TLR stimuli. The inhibition of NF-kB signaling is consistent with our earlier observation in intestinal

epithelial cells (74). We also observed indole-mediated inhibition of the additional pro-inflammatory pathways, Stat3 and Akt. While beyond the scope of this study, it is interesting to note that all three of these pathways are associated with overexpression or persistent activation in multiple human cancers. Further investigation into indole and its potential development for an immunosuppressive anti-cancer agent may be worthwhile. The suppression of multiple ubiquitous pathways would suggest that the cellular target of indole is upstream of common effectors, possibly at the level of TLR signaling. However we have demonstrated that indole does not alter surface TLR4 expression, which leaves the receptor mechanism of indole's effects on DCs to be elucidated.

Our finding that effects of indole on DCs are AhR-independent was unexpected as evidence from the interaction of AhR with host-derived endogenous tryptophan metabolites supports a regulatory role for AhR ligands in DCs. The presence of AhR is required for induction of the host-derived enzyme, indoleamine-2,3-dioxygenase (IDO). This enzyme catalyzes the degradation of tryptophan into kynurenine along with other metabolites. Kynurenine is an endogenous AhR ligand and its addition to DC-T cell co-cultures results in enhanced Treg induction and corresponding inhibition of Th17 differentiation (91). Early exposure of immature DCs to environmental signals determines whether they will express IDO, and IDO- DCs are characterized by immunogenicity whereas IDO+ DCs appear regulatory in nature (120). These IDO+ DCs produce high levels of TGF-β and IL-10 (120), both of which are immunosuppressive and associated with Treg induction. Thus, AhR-dependent tryptophan metabolism by the

host-enzyme IDO produces kynurenine, an AhR ligand which imparts tolerogenic properties upon DCs. These studies using host-derived tryptophan metabolites contrasted with our current work with a microbiota-derived metabolite suggest that AhR activities might be differentially regulated based on origin of the AhR ligand.

In addition, we propose that the ligation and downstream effects of AhR signaling are highly context dependent. Previously we found indole to exhibit antagonist AhR activity in CaCo-2 intestinal cells (83), while both agonistic and antagonistic AhR effects have been previously reported for indole in yeast and a liver cancer cell line, respectively (121, 122). Indeed, both agonistic and antagonistic activities have been identified in numerous AhR ligands as well as tissue/cell-specific activities (123). Indole is a small (MW = 117.15), hydrophobic molecule shown to be freely diffusible across membranes (76). Therefore, indole might bind to either extracellular or intracellular receptors. Future investigation into the binding site of indole will be tremendously advantageous in further characterizing the biological activities of this metabolite.

Due to the largely suppressive nature of the effects of indole on DCs, it is possible that our observations are a result of a general dampening of DC function. Importantly, we provide evidence here that indole does not modify any general antigen-presenting cell functions in DCs. This is important, as inhibition of DC maturation is a mechanism by which factors including IL-10 and TGF-β promote tolerogenic DCs (124). In fact, the long held paradigm has been that immature and semi-mature DCs drive T cell anergy

and Treg formation, whereas fully matured DCs promote immunogenic responses (61). Evidence in recent years has demonstrated unequivocally that fully mature, antigen-loaded DCs are capable of possessing tolerogenic properties (61); thus, factors independent of maturation status are involved in mediating DC tolerance versus immunogenicity. Our findings suggest that indole may be one of these previously unidentified factors.

An intriguing finding in our study is the observation that indole upregulates homing markers on DCs. Indole-conditioned DCs exhibited enhanced CCR7 expression (Figure 28), which indicates a superior capacity to traffic to and enter the lymph nodes. As CCR7 ligands are secreted in T cell areas of lymph nodes (125), we speculate that indole-mediated CCR7 upregulation may augment interactions between DCs and naïve T cells *in situ*.

Similarly, indole conditioning appears to upregulate CCR9 on DCs. Cells expressing CCR9 selectively migrate to its specific ligand, CCL25, produced in the small intestine. Our identification of indole as a suppressor of inflammatory signaling coupled with CCR9 induction support our hypothesis that indole promotes tolerogenic characteristics in DCs. Outside of the mucosa, tissues are largely sterile and thus DCs are highly immunogenic in order to prime a rapid and effective adaptive immune response against invading pathogens. Therefore, it would be logical to confine indole-instructed, tolerogenic DCs to the GI tract where anti-inflammatory properties are essential. This

finding is particularly novel as the current notion is that RA is indispensable for CCR9 upregulation in DCs. Here, we have identified that a single microbiota metabolite is similarly capable of this action. We have shown that the molecular mechanism of action is not enhanced metabolism of RA, hence indole modulates DC properties through a novel mechanism that remains to be defined.

CHAPTER III

INDOLE-CONDITIONED DENDRITIC CELLS MODULATE NAÏVE T CELL ACTIVATION

Overview

In chapter two we have revealed indole as a novel microbiota signal that imparts direct modulation of dendritic cell phenotype (homing markers) and function (cytokine production). Dendritic cells are classified as professional antigen presenting cells, with their predominant function being naïve T cell activation. Thus, our next focus was investigating whether indole conditioning of DCs could modify their interactions with naïve T cells and ultimately modify T cell lineage skew. To this end, we studied DC-T cell interactions and reported the following observations:

1. Indole-conditioned DCs preferentially induce Tregs under both monoclonal and polyclonal stimulation. This effect appears to be AhR-independent and cell contact-dependent.
2. Indole-conditioned DCs induce gut homing CCR9 on naïve T cells.
3. Indole-conditioned DCs inhibit Th1, Th2, and Th17 lineage differentiation.

Rationale

The predominant general function of DCs is their activation of naïve T cells (Figure 32). In addition to providing the MHC-peptide complex and co-stimulatory factors, DCs also secrete cytokines that aid in the lineage determination of the naïve T cell. This phenotype

of the newly activated T cell determines its functional capacity, thus DCs are largely responsible for immune activation versus tolerance. Intestinal DCs play a crucial role in mediating homeostasis in the gut, allowing for the activation of inflammatory responses against pathogens while promoting local immunological tolerance to prevent aberrant inflammation. A major functional feature of gut mucosal DCs is their propensity to induce anti-inflammatory regulatory T-cells rather than pro-inflammatory effector T-cells (55). Based on our previous observations in intestinal epithelial cells (74) and our current observations in DCs, indole appears to suppress pro-inflammatory signals. Thus, we predict that indole will condition DCs to mimic primary mucosal DCs by promoting regulatory T cells. While few host-derived factors such as retinoic acid and TGF-β have been identified as signals that induce Tregs (55), potential remaining signals responsible for conferring mucosal properties to DCs remain to be characterized. Determining whether the microbiota-derived metabolite, indole, can modify DC interactions with T cells in a manner consistent with gut DCs would be a novel finding with enormous therapeutic potential.

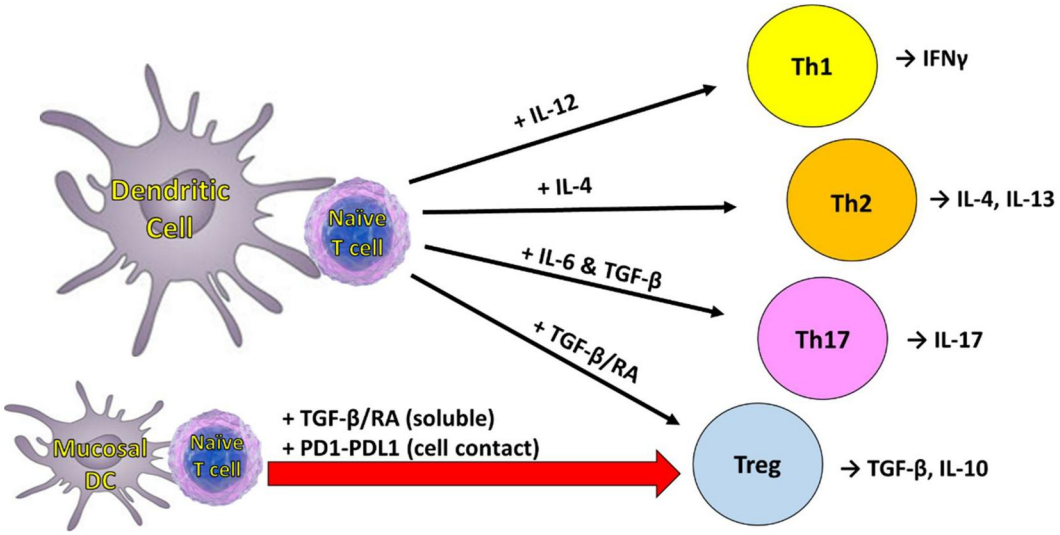

Figure 32. Dendritic cells interact with naïve T cells to determine effector lineage.

Antigen-loaded dendritic cells (DC) activate naïve T cells by presenting antigenic peptide and interacting with co-stimulatory molecules, along with a third signal mediated by soluble factors or cell contact. This signal from the DC, such as cytokine production, determines the effector lineage and resulting downstream function of the newly activated T cell. Mucosal DCs preferentially induce regulatory T cells (Treg) by soluble or cell contact-mediated mechanisms.

Results

Indole-conditioned DCs preferentially induce Tregs under both monoclonal and polyclonal stimulation

To test our hypothesis that indole-conditioned DCs would preferentially induce Tregs, we employed a co-culture system using FACS-sorted wild-type naïve CD4+CD25- T cells. These naïve T cells were activated by antibodies against CD3 and CD28 in the presence of indole-conditioned BMDCs and cultured for 72 hours (see Figure 33 for diagram of experimental design). Under this model of polyclonal stimulation, we observed a 2-fold increase in Treg induction in indole-DC cultures compared to controls (Figure 34). While this finding supports our hypothesis, we chose to pursue the same question in a more physiologically relevant model; i.e. under monoclonal T cell stimulation. To this end, we performed similar experiments using an in vitro antigen-presentation assay with naïve CD4+CD25- T cells from OT-II transgenic mice that express a T cell receptor specific for the peptide sequence 323-339 of chicken ovalbumin (OVA) protein in the context of MHC Class II molecules (Figure 35). Purified naïve CD4+ T cells were co-cultured with OVA323-339 peptide-pulsed BMDCs that were conditioned with indole or retinoic acid from day 3 of development. BMDC preparations were washed extensively to remove any residual conditioning agents. Consistent with our previous finding, indole-conditioned BMDCs preferentially induced Foxp3+ Tregs, an effect dependent upon exogenous TGF-β being added to the cultures (Figure 36). Significant Treg induction by indole-DCs above solvent controls was observed when TGF-β was added at 10 and 30 ng/ml, achieving 3.5-fold greater Treg induction under

these conditions. Significance was not reached when exogenous TGF-β was absent or added at a low concentration (2 ng/ml), which leads us to the conclusion that TGF-β is a necessary co-factor for preferential Treg induction by indole-conditioned DCs.

In order to confirm that the differences observed were due to newly activated T cells, we labeled the naïve T cells with cell proliferation dye prior to co-culturing and tracked its dilution. Similar proliferation was observed across all co-culture conditions with approximately 60% of the initial T cell population undergoing proliferation (Figure 37), thus indicating a similar capacity of antigen presentation by DCs in all groups. This result verifies our conclusion that indole-conditioned DCs enhance Treg induction *ex vivo.*

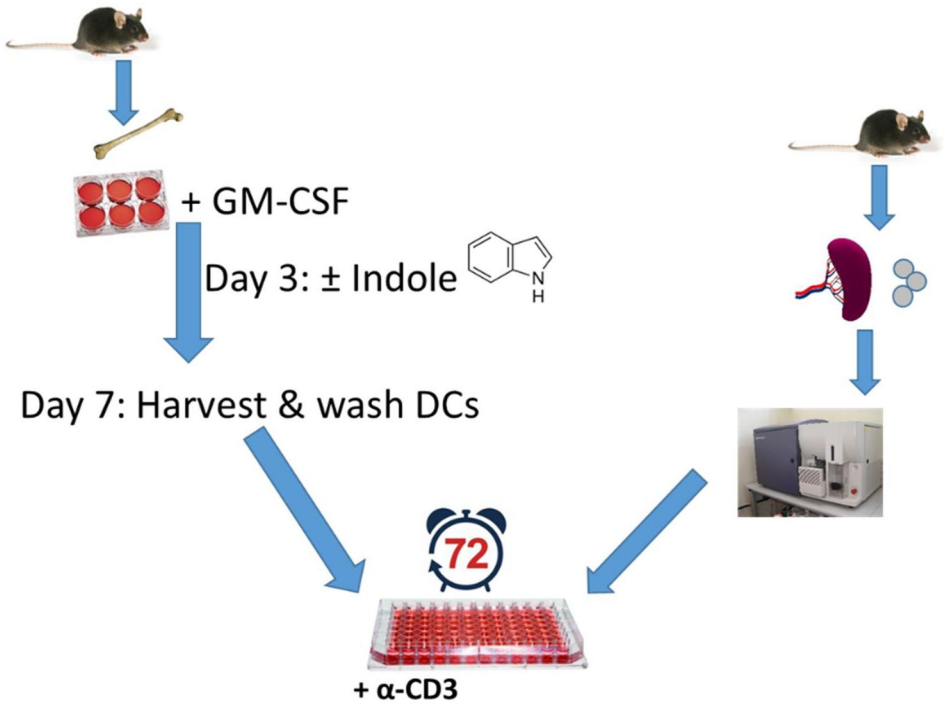

Figure 33. Co-culture system for naïve CD4+CD25- T cell and indole-treated BMDCs (polyclonal stimulation).

Naïve CD4+CD25- T cells from pooled spleen and lymph nodes of C57BL/6 wild-type mice were FACS-sorted to high purity. BMDCs were treated with indole, RA, or DMF from day 3. On day 7, DCs were washed extensively prior to co-culture with the naïve FACS-sorted T cells for 72 hours in a 96-well round-bottom tissue culture plate in the presence of soluble anti-CD3 (5ug/ml). Subsequently, cells were stained for flow cytometric analysis.

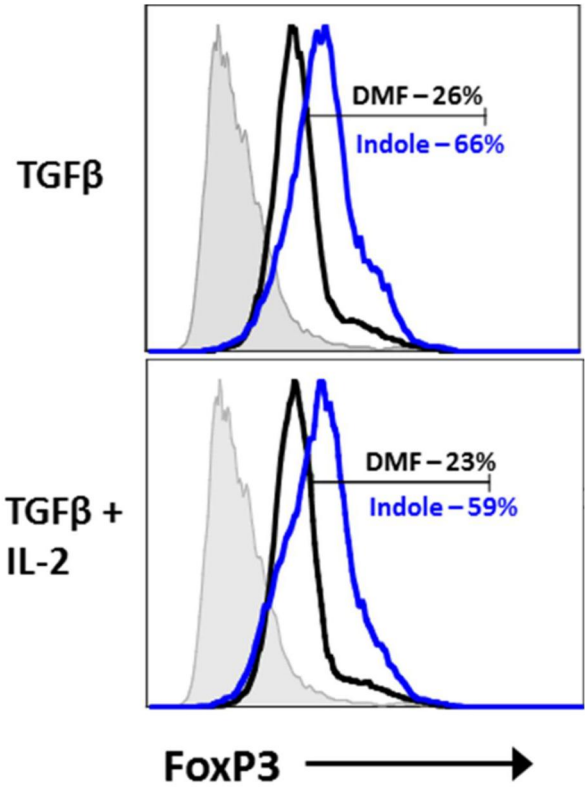

Figure 34. Indole-conditioned DCs preferentially induce Foxp3+ Tregs (polyclonal stimulation).

Naïve wild-type CD4+CD25- T cells were sorted and co-cultured with indole- or DMF-conditioned BMDCs. Cells were cultured together for 72 hours in the presence of soluble α-CD3 (5ug/ml) as well as TGF-β (2 ng/ml) with or without IL-2 (100 U/ml). Subsequently, cells were stained for the transcription factor Foxp3 and analyzed by flow cytometry. Shaded gray histograms represent unstained controls. Representative data from one of four experiments.

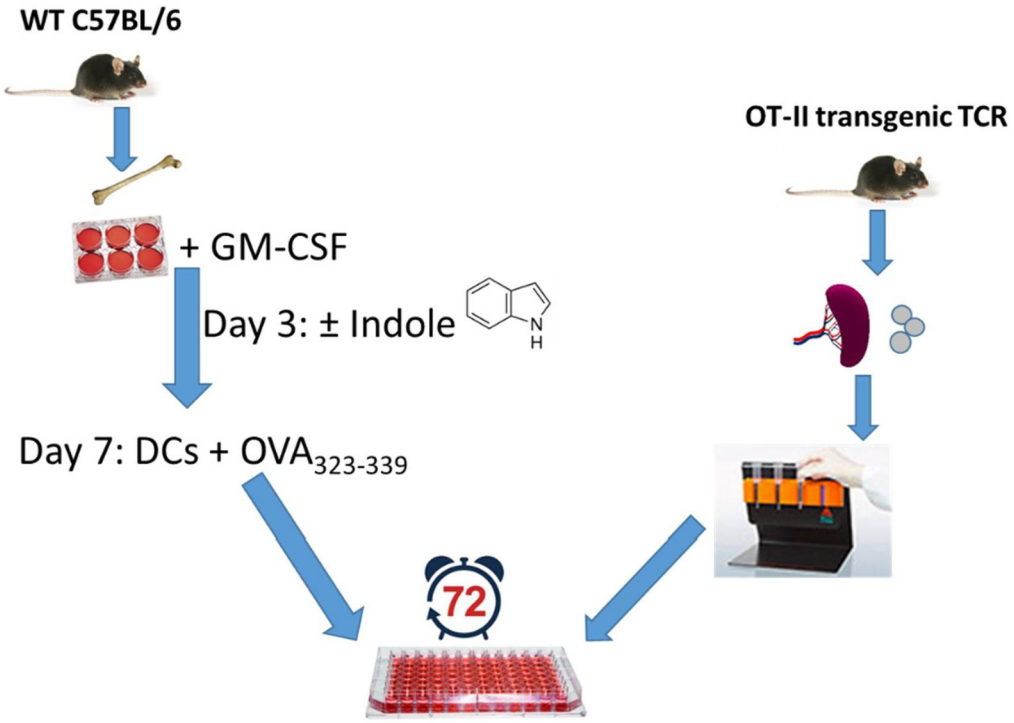

Figure 35. Co-culture system for naïve CD4+CD25- T cell and indole-treated BMDCs (monoclonal stimulation).

Naïve CD4+CD25- T cells of spleen and lymph nodes from OT-II mice were isolated via column purification. T cells were labeled with Cell Proliferation Dye then co-cultured with OVA peptide-loaded BMDCs at a ratio of 2:1 for 72 hours in a 96-well round-bottom tissue culture plate. BMDCs were previously treated with indole or RA from day 3. On day 7, DCs were washed and loaded with $OVA_{323-339}$ peptide (10ug/ml). BMDCs were then washed and plated with naïve CD4+ T cells. Following 3 days of culture, surface and intracellular staining was performed for flow cytometric analysis.

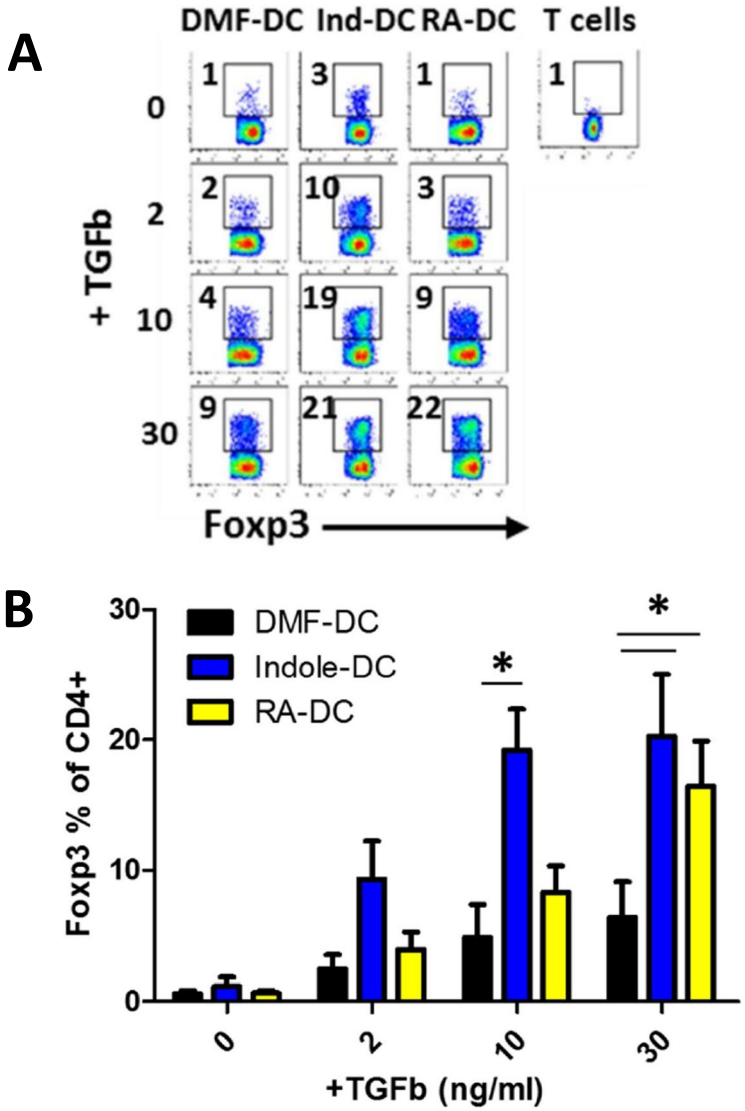

Figure 36. Indole-conditioned DCs preferentially induce Foxp3+ Tregs (monoclonal stimulation).

Naïve CD4+CD25- T cells from OT-II TCR transgenic mice were isolated and labeled with cell proliferation dye. Naïve T cells were then co-cultured with OVA323-339 peptide- loaded BMDCs for 72 hours in the presence of TGF-β as indicated. BMDCs were previously treated with indole, RA, or DMF from day 3. Following 3 days of co-culture, surface and intracellular staining was performed for flow cytometric analysis. (A) Representative plots with numbers indicating the percentage of Foxp3-positive DCs. T cells alone included as negative control. (B) Mean percentage of Foxp3-positive DCs from four independent experiments. * indicates $p < 0.05$ for one-way ANOVA followed by Dunnett's post-test.

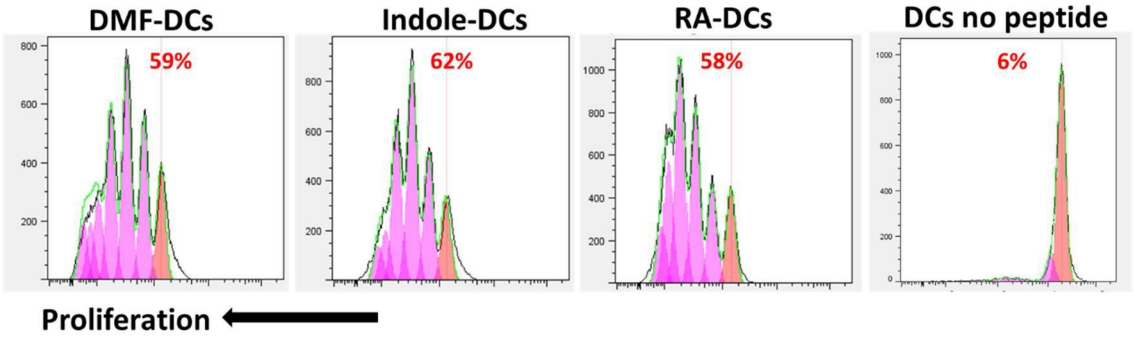

Figure 37. Indole-conditioned DCs induce similar T cell proliferation to control DCs.

Naïve CD4+CD25- T cells were labeled with Cell Proliferation Dye prior to DC co-culturing experiments. After 3 days, dilution of dye was assessed by flow cytometry to determine extent of proliferation. Percentage of actively dividing cells indicated in red. Co-culture with DCs lacking OVA peptide (DCs no peptide) included as negative control of T cell activation. Representative data from one of three independent experiments.

Induction of Tregs by indole-DCs is not mediated by soluble factors

In order to gain insight into the mechanism of indole-mediated Treg skew, we queried whether these effects were mediated via soluble factors. Naïve CD4+ T cells were polyclonally activated by anti-CD3 in the presence of supernatant from indole-treated BMDC cultures. These BMDC supernatants were generated by growing indole-treated BMDCs for seven days, then washing and culturing for an additional 24 hours to generate indole-free supernatant. Retinoic acid-treated BMDC supernatants were included as a positive control and induced Tregs at approximately 2-fold the magnitude of solvent control DCs. No significant induction of Tregs was observed with indole-DC supernatants beyond solvent controls, suggesting that soluble factors are not responsible for indole-DC Treg skew (Figure 38).

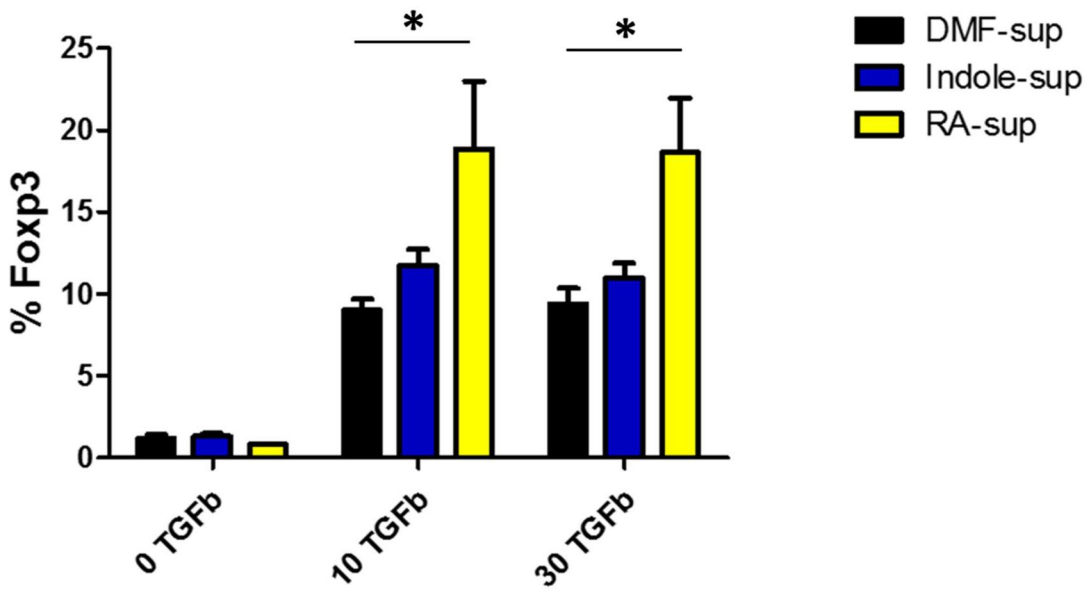

Figure 38. Conditioned media from indole-treated DCs does not induce Treg differentiation.

Naïve CD4+CD25- T cells were FACS-sorted and plated in 96-well, α-CD3 and α-CD28-coated tissue culture plates in a volume of 100 ul cell culture media. An equal volume of spent DC supernatant from Indole, RA, or DMF-conditioned BMDC cultures was added. T cells with DC supernatant were cultured for 72 hours in the presence or absence of exogenous TGF-β. Subsequently, cells were stained for the transcription factor Foxp3 and analyzed by flow cytometry. Mean of three independent experiments. * indicates $p < 0.05$ for one-way ANOVA followed by Dunnett's post-test.

To validate this observation, we employed an additional culturing method. Here, we utilized transwell tissue culture plates to perform co-cultures with a physical barrier between the two cell types. Naïve CD4+ T cells were isolated and placed in the bottom chamber of the transwell plate, activated by plate-bound anti-CD3. Indole- or RA-treated BMDCs were washed and placed in the upper chamber. In this manner, the T cells and DCs share common media and secreted factors in the absence of physical contact. Once again, indole-conditioned DCs did not enhance Treg skew despite the addition of exogenous TGF-β, while positive control RA-DCs enhanced Treg induction 3-fold over solvent controls (Figure 39). Our results confirm the supposition that RA-exposed DCs preferentially induce Tregs via soluble factors. In addition, we conclude that the increased induction of Tregs by indole-DCs does not appear driven by soluble factors but is instead likely mediated by a cell contact-dependent mechanism.

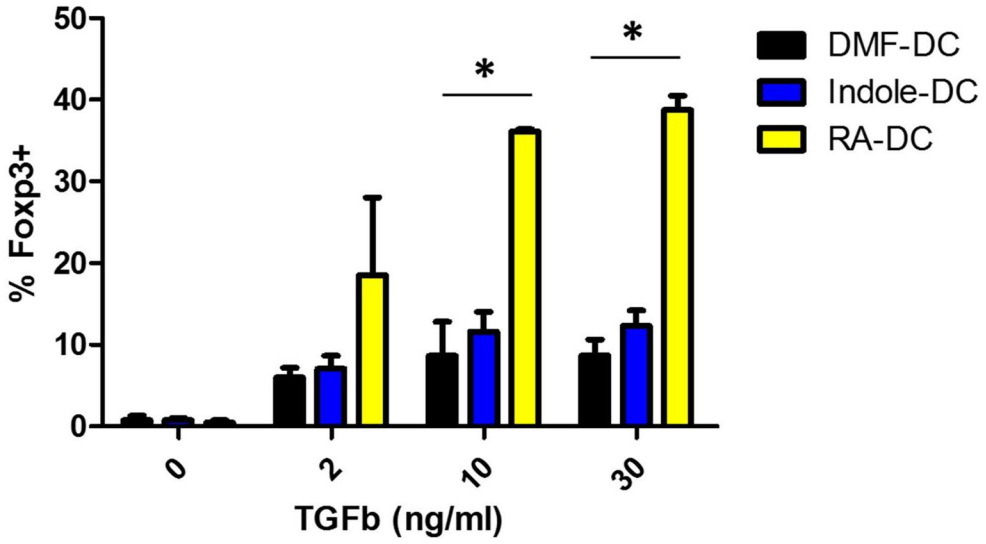

Figure 39. Indole-conditioned DCs do not induce Tregs when physically separated from naïve T cells.

Naïve CD4+CD25- T cells were FACS-sorted and placed in the bottom of a 24-well Transwell cell culture plate with 0.4 uM polycarbonate membranes that had been coated with α-CD3 (5 ug/ml) and α-CD28 (2 ug/ml). TGF-β was added at the indicated concentrations. The insert was placed in the well and indole-, RA-, or DMF-conditioned BMDCs were added to the top chamber such that culture media was shared, but the cell types were kept physically separate. The T cell to DC ratio was 2:1, as in other co-culture experiments. After 3 days, T cells were stained for Foxp3 and analyzed by flow cytometry. Mean of three independent experiments. * indicates $p < 0.05$ for one-way ANOVA followed by Dunnett's post-test.

Indole-conditioned BMDCs induce Tregs in the spleen in vivo

To address the functionality of indole DC-induced Tregs, we employed an *in vivo* T cell activation system (see Figure 40 for schematic representation). The pan T cell surface antigen, Thy1, has congenic alleles, Thy1.1 and Thy1.2, which can be used to differentiate donor and recipient T cells. We purified naïve CD4+CD25- T cells from OT-II (Thy1.2) mice, then transferred them to recipient Thy1.1 mice. We subsequently transferred indole-treated, $OVA_{323-339}$ peptide-loaded BMDCs (Thy1.2) to the same recipient Thy1.1 mice. After five days, we sacrificed the recipient mice to assess activation of the transferred naïve T cells, which could only be activated by the transferred, peptide-loaded DCs and are identifiable by the Thy1.2 T cell surface antigen. We examined cellular populations from the spleen, mesenteric lymph nodes, and pooled distal lymph nodes (axial and inguinal), limiting our analysis to CD4+ T cells bearing the Thy1.2 allele (donor-derived). Importantly, our DCs transferred without OVA peptide loading (negative control) induced virtually undetectable T cell activation. This confirms the specificity of our model, ensuring that T cell activation in Thy1.2 T cells was solely due to interaction with our experimental BMDCs.

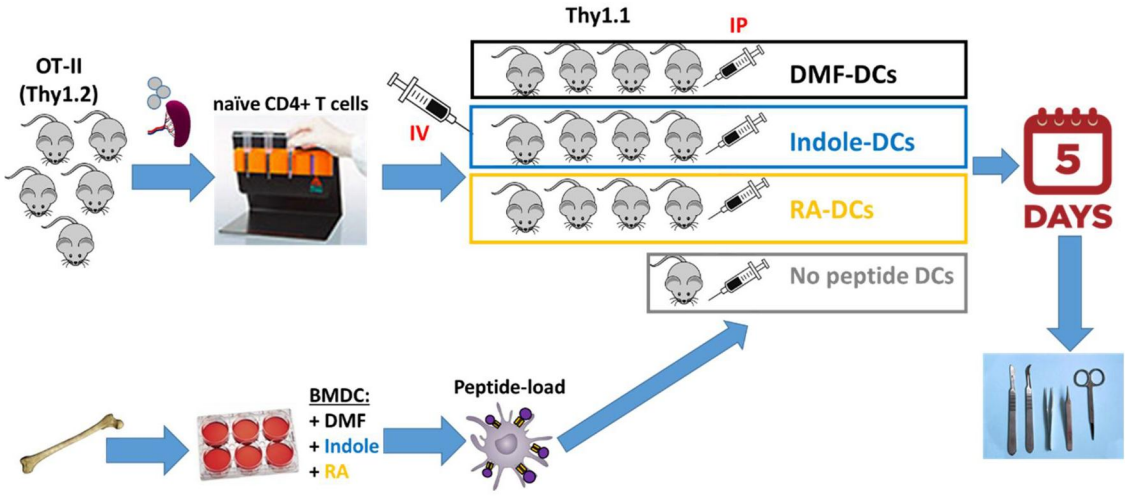

Figure 40. Model of *in vivo* T cell activation by indole-conditioned BMDCs.

Naïve CD4+CD25- T cells from spleens and lymph nodes of OT-II mice were isolated by column purification. Two million T cells were transferred via i.v. injection (retro-orbital route) into recipient B6-Thy1.1 mice. Day 7 BMDCs cultured with indole, RA, or DMF from day 3 were washed and loaded with OVA peptide as described above. Two million BMDCs were transferred i.p. into the same B6-Thy1.1 recipient mice 5 hours subsequent to T cell transfer. After 5 days, tissues were harvested for cellularity analysis as well as *ex vivo* stimulation for cytokine production.

All three tissues demonstrated a substantially lower amount of T cell activation in mice receiving indole-DCs, more than 2-fold less expansion than controls (Figure 41). In fact, T cell expansion induced by indole-DCs was similar to the negative control mice which received DCs lacking OVA peptide. This result was in contrast to our *ex vivo* co-culture work where the extent of T cell activation was unaffected by indole-DCs, indicating that additional physiological factors might modify T cell activation by indole-DCs *in vivo*.

Strikingly, of the newly activated T cell population, indole-DCs induced two times more Tregs in the spleen compared to either RA-DCs or DMF-DCs. In addition to a greater proportion of Tregs, these splenic Tregs had a higher mean fluorescence intensity for the Foxp3 transcription factor, indicative of greater Treg stability and suppressive function (126). This enhancement of Treg induction supports our hypothesis that indole-conditioned DCs promote tolerance *in vivo*.

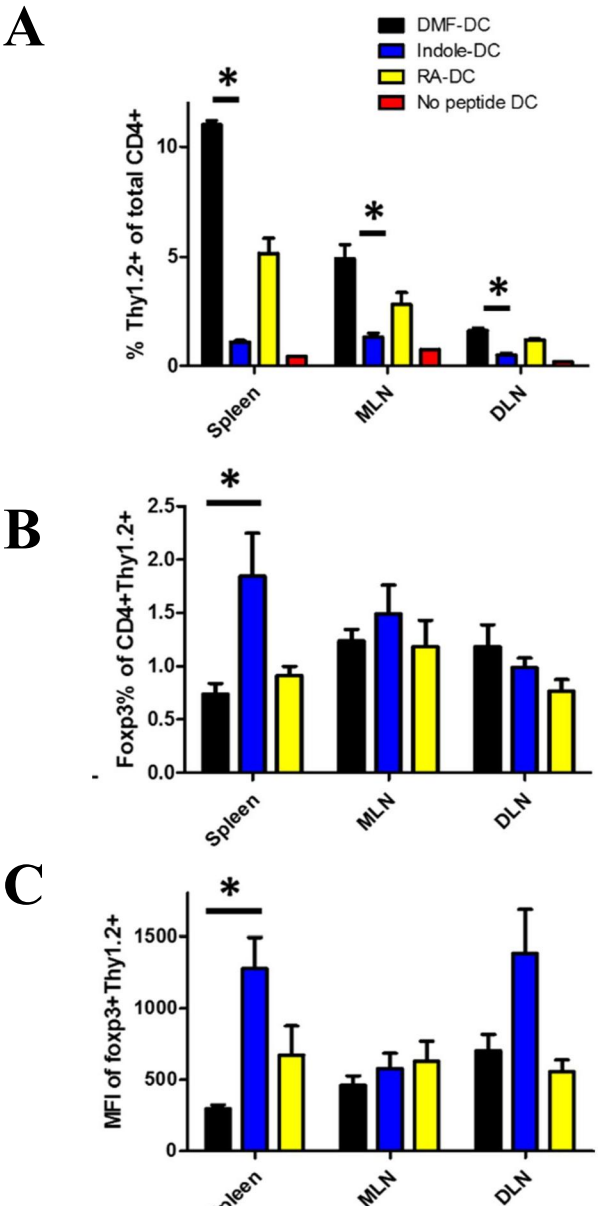

Figure 41. Indole-conditioned DCs induce lower T cell activation overall but a greater proportion of Tregs in spleen compared to control.

Naïve CD4+CD25- T cells from OT-II (Thy1.2) mice and $OVA_{323-339}$-loaded BMDCs conditioned with indole, RA, or DMF were transferred to B6-Thy1.1 mice. After 7 days, tissues were harvested and stained for flow cytometric analysis. Data includes (A) total expansion of Thy1.2+CD4+ T cells, (B) Percentage of Foxp3+CD4+ T cells of expanded T cells, and (C) MFI of Foxp3+CD4+ T cells of expanded T cells. n = 4 mice/group. One representative experiment of two shown. * $p < 0.05$ by Student's *t* test.

Indole-conditioned DCs induce gut homing CCR9 on naïve T cells

Based on our observation that indole induces gut homing markers on developing DCs, we decided to investigate whether indole-conditioned DCs could also confer gut homing markers upon naïve CD4+ T cells. We used our antigen-specific T cell activation model to address this question, and a titration of RA was included as a positive control to induce CCR9. We found that while indole-conditioned BMDCs alone were insufficient to induce CCR9 on naïve T cells, the addition of RA to these cultures resulted in significant CCR9 induction above solvent controls (Figure 42). Just 10 nM of RA enabled indole-DC co-cultures to achieve over 50% of T cells expressing CCR9 which was similar to RA-DC positive controls, whereas DMF-DC co-cultures with the same concentration of RA added achieved only 10% of CCR9+ T cells. Throughout our titration of RA concentrations ranging 10-1000 nM, indole-DCs consistently induced a similar level of CCR9 expression on T cells compared to RA-DCs and a greater level than was achieved in DMF-DC controls. This data allows us to conclude that RA is an essential co-factor for preferential induction of gut-homing CCR9 on naïve T cells by indole-conditioned DCs. The functional significance of this upregulation has been demonstrated *in vivo*, with newly activated T cells bearing CCR9 effectively migrating to the small intestine (46, 119).

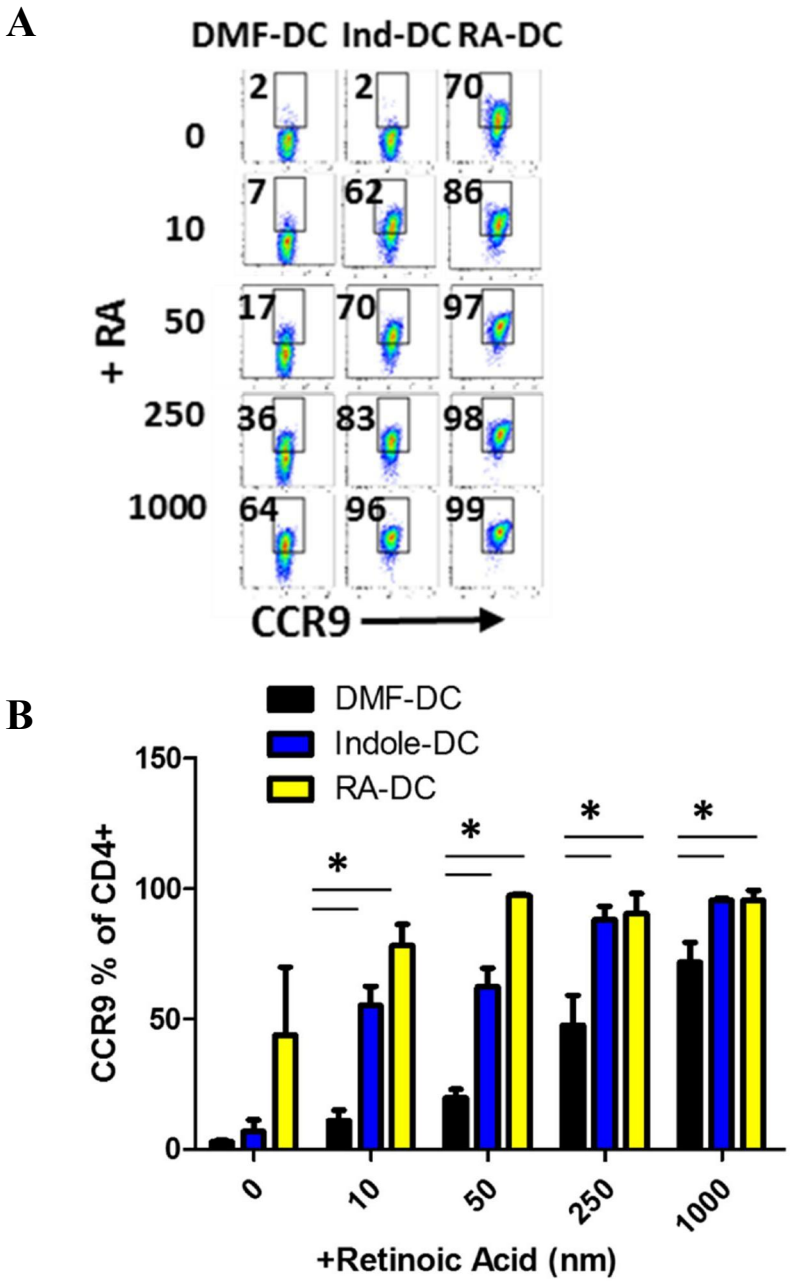

Figure 42. Indole-conditioned DCs induce CCR9 on naïve T cells upon activation.

Naïve CD4+CD25- T cells from OT-II mice were co-cultured with $OVA_{323-339}$ peptide-loaded BMDCs for 72 hours in the presence of RA as indicated. BMDCs were previously treated with indole, RA, or DMF from day 3. Following 3 days of co-culture, surface staining was performed for flow cytometric analysis. (A) Representative plots indicating percentage of CCR9+ of total CD4+ cells. (B) Mean of three independent experiments. * indicates $p < 0.05$ for one-way ANOVA followed by Dunnett's post-test.

Indole-conditioned DCs inhibit Th1, Th2, and Th17 lineage differentiation

In order to determine whether the indole-conditioned DC induction of Tregs was specific to this lineage, we needed to query additional T cell fates. Activated CD4+ T helper cells are typically divided into the subsets Th1, Th2, Th17, and Treg, based on their expression of transcription factors and pattern of cytokine secretion. We used our antigen-specific co-culture system to investigate these remaining traditional T cell lineages with the addition of an endpoint PMA/Ionomycin stimulation to induce cytokine secretion. We first examined Th17 induction, as concurrent work in this lab has suggested that conditioning of naïve T cells directly with indole in the absence of antigen-presenting cells inhibits Th17 differentiation (Steinmeyer et al, in preparation).

In agreement with these observations, we found that indole-conditioned DCs also inhibit Th17 differentiation, despite the presence of Th17-skewing cytokines added to the culture (Figure 43). The presence of indole-DCs maintained Th17 differentiation at a rate of less than 0.5% of T cells, compared to 2.5% Th17 in DMF-DC control cultures. Importantly, this indicates that indole conditioning of DCs is able to overcome physiological skewing factors which drive Th17 induction. Next, we interrogated the Th1 and Th2 lineages that largely mediate cell-mediated and humoral immune responses, respectively. Once again, we observed a stark inhibition of the development of these lineages (Figure 43). These findings indicate that indole conditions DCs to preferentially induce regulatory T cells while suppressing the differentiation of other, largely pro-inflammatory, helper T cell lineages.

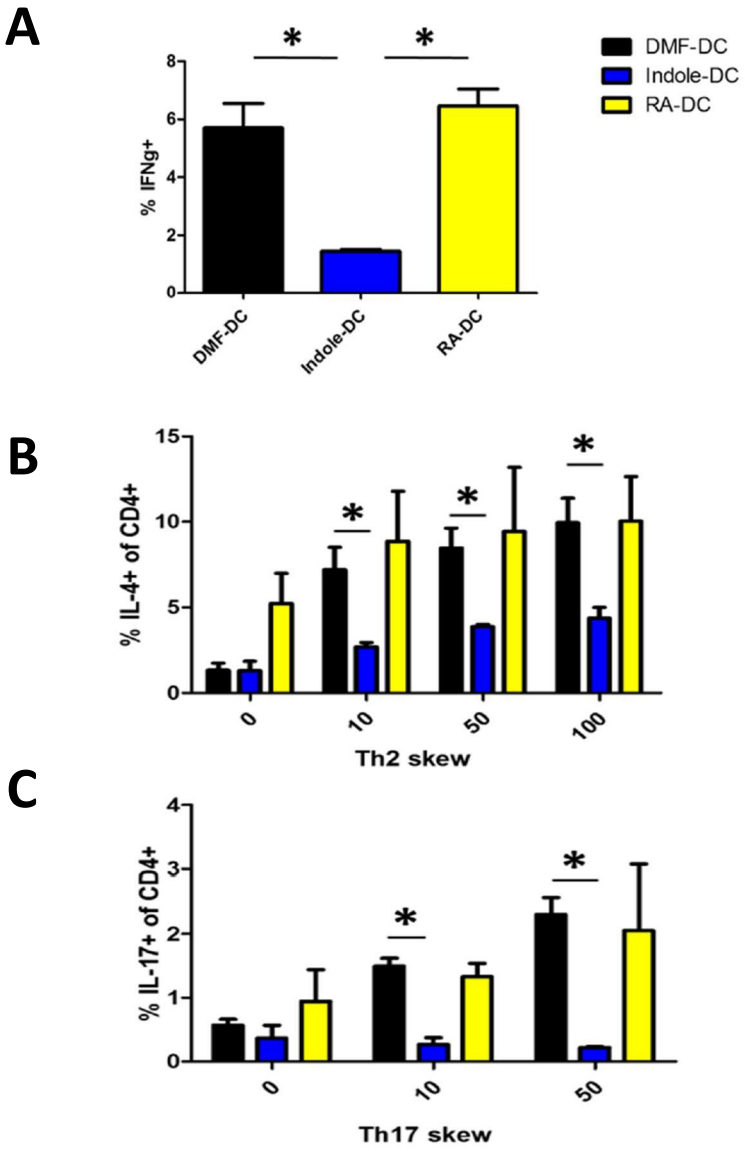

Figure 43. Indole- conditioned DCs inhibit Th1, Th2, and Th17 differentiation.

Naïve CD4+CD25- T cells from OT-II TCR transgenic mice were isolated by column purification. Naïve T cells were then co-cultured with $OVA_{323-339}$ peptide-loaded BMDCs for 72 hours in the presence or absence of lineage-skewing cytokines (described in methods). BMDCs were previously treated with indole, RA, or DMF from day 3. For the last 5 hours of culture, T cells were restimulated with PMA and ionomycin in the presence of Golgiplug, followed by intracellular staining. Cytokine production was determined to assess differentiation of (A) Th1, (B) Th2, and (C) Th17 CD4+ T cells. Mean of three independent experiments. * indicates $p < 0.05$ for one-way ANOVA followed by Dunnett's post-test.

T cell lineage skewing by indole-DCs is AhR-independent

Using a similar co-culture system, others have reported that AhR-deficient BMDCs skew naïve T cell differential away from Tregs (91). Because we have observed skewing towards Tregs by indole and we have identified indole as an AhR ligand (83), we sought to determine whether Treg skew by indole might be mediated via the AhR pathway. We performed antigen-specific T cell activation using naïve T cells from OT-II mice co-cultured with indole-treated BMDCs derived from wild-type or AhR-/- mice. Consistent with the findings from Nguyen and associates, (91), we observed an overall decrease in Treg induction (~30%) when naïve T cells were activated with AhR-/- BMDCs. However, similar to our results in chapter 2, we found that the Treg skewing induced by indole-conditioned BMDCs was entirely independent of AhR (Figure 44). Similarly, the inhibition of the Th1 lineage by indole-conditioned BMDCs was apparent in co-cultures using DCs derived from both wild-type and AhR-deficient mice, resulting in our conclusion that this effect of indole is also AhR-independent (Figure 44).

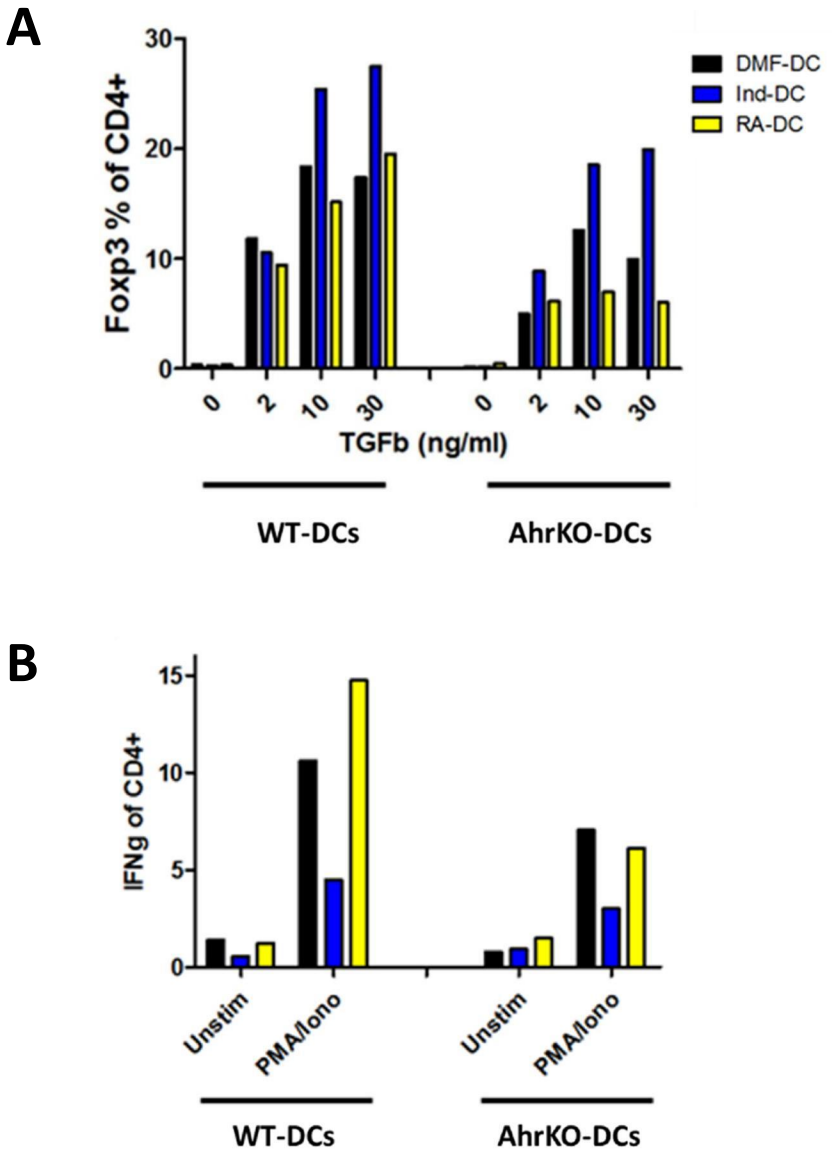

Figure 44. T cell skewing mediated by indole-conditioned DCs is AhR-independent.

BMDCs were differentiated from WT or AhR-KO mice and conditioned with indole, RA, or DMF from day 3. DCs were then loaded with $OVA_{323-339}$ and co-cultured with naïve CD4+CD25- T cells from OT-II transgenic mice for 72 hours. For Th1 cultures, T cells were restimulated with PMA and ionomycin in the presence of golgiplug for the last 5 hours of culture. Subsequently, all cultures were stained and analyzed by flow cytometry for (A) Foxp3 to identify Tregs or (B) IFNγ to identify Th1 cells.

Transfer of indole-conditioned DCs did not protect against DSS-induced colitis

Based on our findings that indole-conditioned DCs inhibit pro-inflammatory responses and preferentially induce Tregs *in vivo*, we predicted that transferring these DCs prior to the induction of DSS-induced colitis would lessen the severity of colitis in recipient mice. This concept has been applied previously with the transfer of Polysaccharide A (PSA)-treated BMDCs to mice prior to the induction of TNBS colitis (73, 127). Both groups reported lower clinical disease scores in mice to which PSA-treated BMDCs were adoptively transferred prior to colitis induction, attributed to both innate and adaptive mechanisms. We chose to use the dextran sodium sulfate (DSS) model of murine colitis based on the robustness and reproducibility of the model as well as its similarities to inflammatory bowel disease in humans, particularly ulcerative colitis.

BMDCs were cultured with indole, RA, or DMF from day 3 as described above and transferred to wild-type mice on days 0, 6, and 9 of the experiment. Mice received 5% DSS in their drinking water from day 7 to day 14 and were euthanized on day 20 for subsequent tissue harvest and processing. Bodyweights of individual mice were tracked as an indirect measure of clinical disease. As expected, weight loss peaked a few days following the removal of DSS upon which mice began to recover and gain weight. Throughout the duration of the experiment, no difference in bodyweight was observed between mice receiving indole-treated DCs compared with solvent control-treated DCs (Figure 45). Similarly, differences were not apparent between these two groups for the following T cell parameters in either spleen or mesenteric lymph nodes: CD4+ T cells,

CD8+ T cells, Foxp3+ Tregs, Th1 cells, and Th17 cells (Figure 46). Analysis of antigen-presenting cells in these tissues also revealed no differences between indole-DC and DMF-DC mice for the presence of total MHC-II+ APCs, macrophages, DCs, and CD103+ DCs (Figure 46). These results might indicate that under the current experimental conditions, a significant shift in immune cell populations in the spleen or mesenteric lymph nodes did not occur. An alternative possibility is that any changes in immune cellular subsets had been resolved by the time of tissue harvest on day 20.

A hallmark pathological sign of colitis is shortening of the colon, mediated by abscesses forming in the intestinal crypts that disrupt the mucosal architecture and are later replaced by scar tissue. An intriguing finding in our study was that the average colon length of indole-DC-transferred mice was similar to unmanipulated control mice (~6 cm), while DMF-DC-transferred and no DC-transferred mice exhibited shorter colons (~4.5 cm) at endpoint analysis (Figure 47). Shortening of the colon is an established indicator of colitis pathology in the murine model of DSS colitis (128). Thus, our observation suggests that indole-DCs might have played some role in mediating recovery from colitis that was not detected in our experimental readouts.

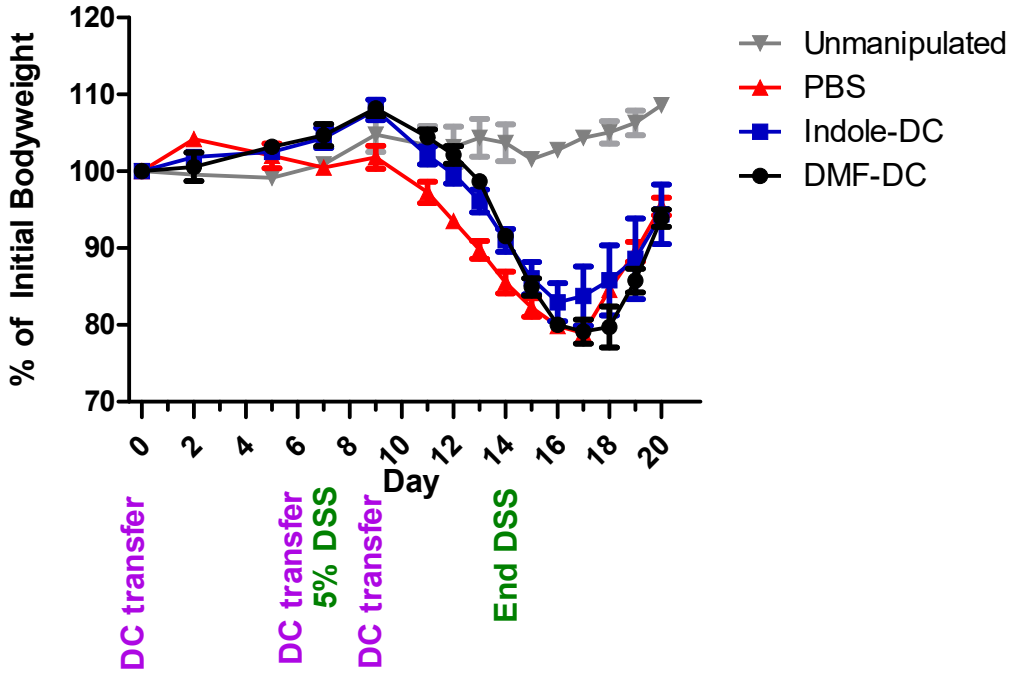

Figure 45. *In vivo* transfer of indole-conditioned DCs did not affect weight loss in a murine DSS colitis model.

BMDCs were cultured with indole, RA, or DMF from day 3. Two million BMDCs were transferred via i.p. injection to WT C57BL/6 mice on days 0, 6, and 9 of the experiment. Mice received 5% dextran sulfate sodium (DSS) *ad libitum* in their drinking water from day 7 through day 14. Mice were weighed daily to monitor weight loss. Mice were euthanized on day 20 for subsequent tissue harvest and processing. n = 4 mice/group.

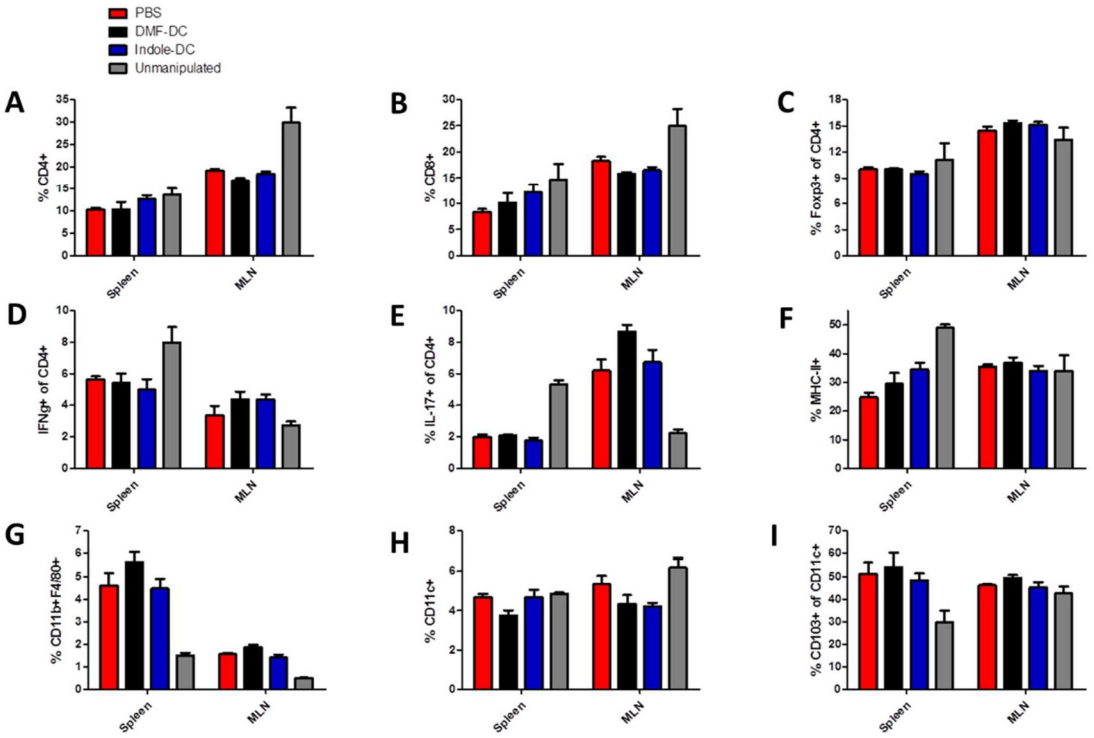

Figure 46. *In vivo* transfer of indole-conditioned DCs did not affect spleen or MLN cellularity in a murine DSS colitis model.

BMDCs were cultured with indole, RA, or DMF from day 3. Two million BMDCs were transferred via i.p. injection to WT C57BL/6 mice on days 0, 6, and 9 of the experiment. Mice received 5% dextran sulfate sodium (DSS) *ad libitum* in their drinking water from day 7 through day 14. Mice were euthanized on day 20 for subsequent tissue harvest and cellularity analysis as well as *ex vivo* stimulation for cytokine production. Unmanipulated mice served as a negative control. n = 4 mice/group. No differences were induced by transfer of indole-conditioned DCs (one-way ANOVA followed by Dunnett's post-test).

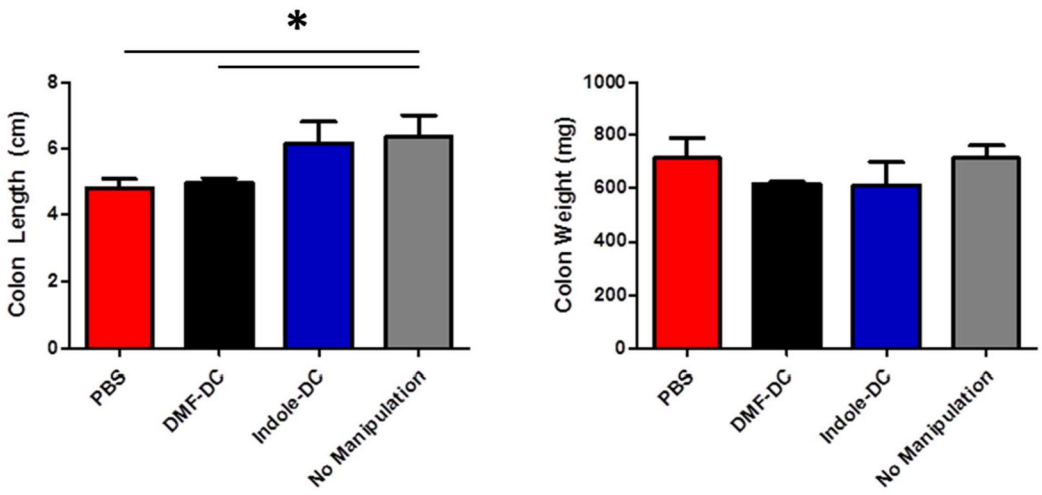

Figure 47. *In vivo* transfer of indole-conditioned DCs prevented shortening of colon in a murine DSS colitis model.

BMDCs were cultured with indole, RA, or DMF from day 3. Two million BMDCs were transferred via i.p. injection to WT C57BL/6 mice on days 0, 6, and 9 of the experiment. Mice received 5% dextran sulfate sodium (DSS) *ad libitum* in their drinking water from day 7 through day 14. Mice were euthanized on day 20 and colons were immediately removed and measured for length (left panel) and weight (right panel). n = 4 mice/group. * $p < 0.05$ by one-way ANOVA followed by Dunnett's post-test, comparing to unmanipulated control colons.

Discussion

In this chapter, we have identified previously unappreciated biology in which conditioning of dendritic cells with a single microbiota-derived metabolite is sufficient to modulate its T cell activating functions in a tolerance-promoting manner (Figure 48). We demonstrated that indole-conditioned DCs preferentially induce Foxp3+ Tregs in a TGF-β-dependent manner, further suggesting a synergistic effect of indole and TGF-β as observed in chapter two. This effect was validated in multiple models of naïve T cell activation- under both polyclonal (anti-CD3) and monoclonal (antigen-specific OT-II) stimulation. The monoclonal stimulation conditions are particularly relevant as this mimics true physiological interaction between a naïve T cell and antigen-presenting cell. Two signals are essential for a naïve CD4+ T cell to become activated by an antigen-presenting cell: First is engagement of the T cell receptor (TCR) by MHC-II on the APC, and second is co-stimulation, typically ligation of T cell CD28 by CD80 or CD86 on the surface of the APC. We have demonstrated in chapter two that indole does not modify general DC function, nor expression of the DC surface molecules MHC-II, CD80, or CD86. This evidence suggests that conditioning of DCs with indole leaves the TCR-APC complex intact and does not account for observed differences in T cell activation.

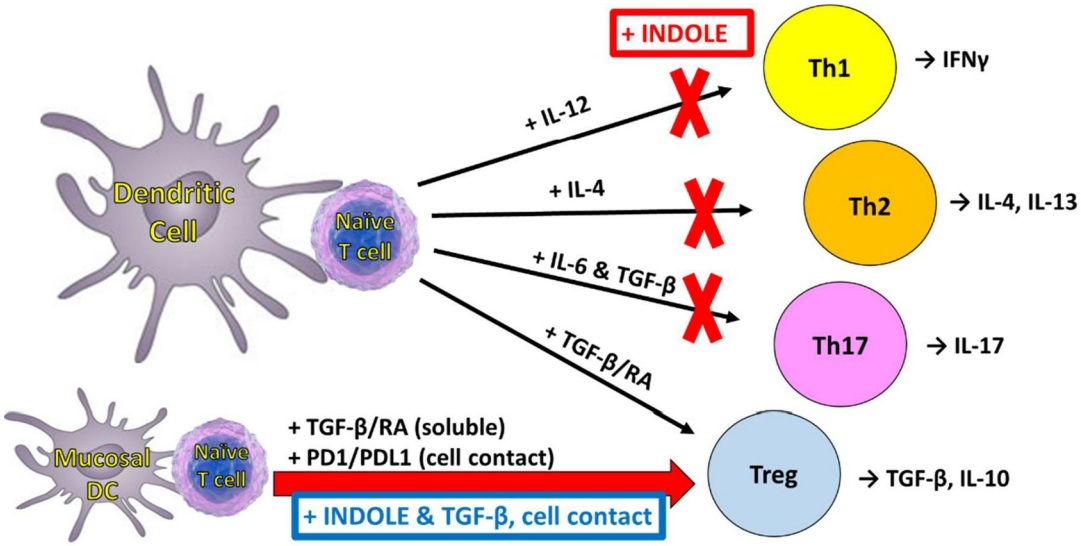

Figure 48. Indole-conditioned DCs preferentially induce Tregs and inhibit Th1, Th2, and Th17 lineages.

Antigen-loaded dendritic cells (DC) activate naïve T cells by presenting antigenic peptide and interacting with co-stimulatory molecules, along with a third signal mediated by soluble factors or cell contact. This signal from the DC, such as cytokine production, determines the effector lineage and resulting downstream function of the newly activated T cell. Mucosal DCs preferentially induce regulatory T cells (Treg) by soluble or cell contact-mediated mechanisms. The current study provides evidence that indole-conditioned DCs preferentially induce Tregs in a TGF-β-dependent manner and requires cell contact between the DC and naïve T cell. In addition, indole-treated DCs inhibited differentiation of Th1, Th2, and Th17 cells.

Our group has previously published on our identification of indole as a ligand of the aryl hydrocarbon receptor (83), thus we acquired AhR-/- mice to pursue the question of whether AhR ligation is the mechanism of action for indole's effects on immune cells. In addition, we have revealed that the presence of indole during anti-CD3-mediated naïve T cell activation also preferentially induces Foxp3+ Tregs. However, studies comparing naïve T cells derived from wild-type versus AhR-/- mice indicated that this effect was at least partially mediated through the aryl hydrocarbon receptor (Steinmeyer et al, in preparation). Therefore it was somewhat surprising to observe that in the current study, indole-DC conditioning effects on T cells were entirely AhR-independent, despite having observed AhR-independent effects of indole directly on DCs in chapter two. Based on these seemingly contradictory observations, we must consider that indole might interact with different immune cell subsets by distinct mechanisms. As discussed in chapter two, both agonistic and antagonistic activities have been identified in numerous AhR ligands as well as tissue/cell-specific activities (123). Further work is warranted to determine the molecular mechanism responsible for indole's specific effects on DCs.

We demonstrated using multiple methods that the observed preferential Treg skew by indole-DCs requires contact between DCs and naïve T cells. This was an unexpected finding, given that the known Treg-skewing elements are soluble factors secreted by host immune and epithelial cells. The host-derived cytokine, TGF-β, is present at high concentrations in the GALT and is produced by gut epithelial cells as well as various

immune cell subsets. This cytokine is well appreciated for its crucial role in intestinal homeostasis; TGF-β-deficient mice develop lethal gut and systemic inflammation (104) and murine models of colitis are alleviated by TGF-β treatment (129, 130). TGF-β is a crucial factor for Foxp3+ Treg induction (131, 132). In a co-culture system similar to ours using naïve T cells and CD103+ DCs isolated from murine MLNs, the addition of a blocking monoclonal antibody against TGF-β completely abrogated Foxp3+ Treg induction (55). Interestingly, authors of this work reported that the addition of exogenous TGF-β to these co-cultures induced a strong enhancement of Treg conversion in cultures with CD103+ DCs but only a minimal induction with CD103- DCs (55).

When we compare these findings with our current study, it is tempting to speculate that conventional BMDCs act similarly to CD103- primary DCs while our indole-conditioned DCs exhibit characteristics that mimic mucosal CD103+ DCs. This is especially intriguing considering that indole did not induce the expression of CD103 on BMDCs *in vitro*; however, it has been suggested that additional physiological factors may be required for CD103 induction (110). A transcriptomic approach would be beneficial in the future to compare the genetic signatures of indole-conditioned BMDCs to bona fide mucosal DCs.

Consistent with the literature, our retinoic acid-conditioned DCs enhanced Foxp3+ Treg skew when supplemented with exogenous TGF-β (55). This supports the notion that both TGF-β and retinoic acid are essential components for Treg induction. Indeed, the

inclusion of synthetic RA inhibitors (LE540 and LE135) blocked the ability of CD103+ MLN DCs to skew naïve T cells towards Foxp3+ Tregs (55), emphasizing the necessity of RA in this conversion.

In addition to its role as a co-factor in Treg induction, retinoic acid also enhances the expression of chemokine receptor CCR9 and the integrin α4β7 on T cells upon activation (53). Retinoic acid is a product of the catabolism of dietary vitamin A, formed by intracellular oxidative metabolism of retinol (133). The major physiological form, all-*trans*-RA, binds predominantly to members of the nuclear retinoic acid receptor family (types α, β, and γ) (134). We found that *in vitro* conditioning with RA during development was sufficient for BMDCs to induce CCR9 on naïve T cells, whereas indole-conditioned BMDCs and control BMDCs did not induce CCR9. However, the addition of exogenous RA into the indole-DC co-cultures resulted in a pronounced induction of CCR9, similar to the level observed in RA-DC cultures. This finding suggests that indole conditioning does not confer RA-producing capacity to DCs, which is in agreement with our unexpected finding in chapter two that indole inhibits aldh1a2 expression and does not induce aldehyde dehydrogenase activity. We instead propose that indole affects DCs through an alternative mechanism that remains to be identified. Based on the findings presented in this chapter, one hypothesis might be that indole primes DCs to exhibit heightened sensitivity to the presence of TGF-β and/or retinoic acid, thus explaining the observed synergistic effects. We propose that binding assays

would be useful to reveal the site of action for indole in DCs in order to help clarify this remaining question.

Our *in vivo* T cell activation experiments affirm our *ex vivo* findings that indole-conditioned DCs are capable of preferentially inducing Foxp3+ Tregs. We observed this Treg enhancement in the spleens of recipient mice, both in proportion of Tregs and Foxp3+ mean fluorescence intensity. Using our *ex vivo* co-culture system we identified exogenous TGF-β as an essential co-factor to direct indole-mediated Treg skew; however our observation of *in vivo* Treg skew indicates that any required co-factors were present and biologically available.

While we had predicted an increase in Tregs in the MLNs as well, indole-DCs did not induce Tregs above solvent control-DCs in this tissue. When contemplating this result, it is important to consider that our recipient mice were conventionally-raised, specific pathogen-free animals and thus colonized by indole-producing bacteria. These mice would have already possessed endogenous DCs conditioned by indole in the MLNs, and we speculate that this may account for the lack of effects observed in the MLNs when transferring indole-DCs. In contrast, we have found indole to be present at trace levels in the blood and virtually undetectable in peripheral organs such as the lungs (unpublished observations); thus we assume the spleen to be an indole-naïve organ. This would indicate that our transfer experiments introduced indole-conditioned DCs to the spleen and this first encounter preferentially induced Tregs.

An important extension of this work would be to repeat in mice lacking indole and indole-producing bacteria. One way to accomplish this would be by using germ-free mice; however, the importance of the intestinal microbiota for a fully functional and developed immune system has been well established, and thus using germ-free mice would introduce additional variables to the system. The ideal approach would be to colonize germ-free mice with a known population of non-indole-producing commensal bacterial strains. In this manner, mice would possess normal, fully functional immune systems and the effects of adding indole to the system could be addressed. An additional set of experiments might include colonizing two groups of mice with a known set of commensal bacterial strains, where one group receives mutant strains of the same bacteria that have the enzyme, tryptophanase, knocked out. Because this enzyme that catalyzes the indole-producing reaction has been identified (135), this approach is feasible and would allow for the comparison of immune cell phenotype and function in the presence or absence of endogenous indole.

An additional consideration for this *in vivo* T cell activation work is that the study was performed under homeostatic conditions. That is, naïve T cells and indole-conditioned DCs were transferred to recipient mice in the absence of any infectious or inflammatory insult. It is possible that this might explain the perceived lower T cell activation observed in mice receiving indole-conditioned DCs. However, a possible explanation might be that the indole-conditioned DCs and/or newly activated T cells migrated to a tissue not included in the current study. Based on the indole-induced upregulation of the

CCR9 gut homing marker, one possibility is that the T cells activated by indole-DCs have migrated to the lamina propria. Further studies should be expanded to include examination of the lamina propria and additional tissues to address the question of lower activation versus T cell migration induced by indole-DCs. Alternatively, while we did not observe this effect in our co-cultures, indole might serve as a general suppressor of T cell activation *in vivo* in the absence of inflammatory stimuli. This overall dampening would limit the activation of all lineages, including inflammatory effector T cell subsets. Investigating additional T cell lineages would be useful; based on our *ex vivo* work, we predict that indole-DCs selectively inhibit Th1, Th2, and Th17 CD4+ T helper cell lineages.

Our observations led us to test the ability of indole-conditioned DCs to alleviate colitis severity *in vivo*. We predicted that under inflammatory conditions, indole-conditioned DCs would inhibit the development of inflammation through both the dampening of pro-inflammatory cytokine production and the preferential induction of regulatory T cells. However, under these experimental conditions, we did not observe a protective effect of indole-DC transfer. There are a number of potential explanations for the lack of protection. One likely possibility is that our dose of DSS, 5% for 7 days, was too high in this experimental setting and caused a level of severe damage to the intestinal epithelium that could not be overcome by potential effects of indole-DCs. To address this, we would optimize DSS conditions to determine ideal dose and duration of DSS. Our DSS procedure of a relatively high dose (5%) for 7 days mimics a more acute disease bout,

whereas multiple cycles of a lower DSS concentration have been shown to resemble chronic intestinal inflammation and might be more relevant for our future experimental questions. Early characterization of DSS effects in mice revealed that an acute treatment results in colonic mucosal inflammation, weight loss, and bloody diarrhea, whereas three to five cycles of DSS induced chronic inflammation with severe mononuclear cell infiltration and regenerative changes in the epithelium (136).

In this study, we have demonstrated that indole inhibits DC production of IL-6. While this cytokine is typically considered to be a pro-inflammatory mediator, it also has an important role as a reparative factor in wound healing (137). Thus, one explanation might be that under homeostatic conditions, indole promotes an anti-inflammatory, tolerogenic environment while in times of tissue injury, the effect of indole is overridden by the need for IL-6 and other wound repairing factors.

Other considerations include the details of the DC transfer: ie, number of DCs transferred and timing of transfers. We transferred two million BMDCs, which is the same number as in the seminal PSA-BMDC transfer paper but considerably higher than the 100,000 and 600,000 BMDC doses transferred in the subsequent publication (73, 127). The duration of DC life *in vivo* should be considered, as impaired indole-DC survival would lessen interaction with T cells. Indeed, the Shen group transferred PSA-BMDCs only two hours prior to TNBS colitis induction (73). Rapid DC turnover *in vivo* has been documented, with lifespan differences reported for different DC subsets (138).

In vivo half-life studies comparing indole-treated and control DCs would be useful to address possible differences in lifespan.

Lastly, we might consider pursuing alternative rodent models of colitis. The protective effects of transferring PSA-loaded BMDCs were observed using the TNBS model (73, 127) in which colitis develops as a delayed-type hypersensitivity response to haptenized proteins (139), whereas DSS colitis develops as a direct result of intestinal barrier injury (140). A comparative gene expression analysis of common mouse models of colitis suggests that the CD45RB transfer model, which involves transferring a T cell population devoid of regulatory cells into an immunodeficient host (141), best resembles human inflammatory bowel disease (142). The incorporation of multiple colitis models into future studies may be useful to screen for potential benefits of indole-DCs in models with varying pathologies, potentially shedding light on the molecular mechanism of action for indole.

CHAPTER IV

PRO-INFLAMMATORY SIGNALING IS DOWN-REGULATED IN INDOLE-CONDITIONED MACROPHAGES

Overview

In addition to dendritic cells, macrophages are the other prominent mononuclear phagocyte residing within the intestinal environment. While the primary role of DCs is activation of naïve T cells, macrophage functions are centered around clearance of bacteria from the tissue and communicating danger signals to other immune cells. To determine whether indole modulates the function of other antigen-presenting cells in addition to DCs, we tested several of our hallmark observations in bone marrow-derived and primary splenic macrophages. The completion of this abbreviated study has revealed these findings:

1. Indole pre-conditioning suppresses pro-inflammatory cytokine production in macrophages.
2. Pro-inflammatory signaling pathways are inhibited by indole conditioning in macrophages.
3. The effects of indole on macrophages appear to be AhR-independent.

Rationale

Macrophages are the most prevalent mononuclear phagocytes in the steady-state lamina propria and play a crucial role in tissue homeostasis (47). The ontogeny of intestinal macrophages is unique from other tissue-resident macrophages in that these cells display a short half-life, under constant renewal from circulating monocytes (143). We predict that these macrophage precursors encounter high concentrations of indole upon entering the intestinal environment, thus indole might play a role in shaping their inflammatory functions. Others have previously demonstrated that treatment of macrophages with the microbial metabolite, butyrate, can alter their function, primarily the down-regulation of pro-inflammatory mediators (70). Our findings in chapter two revealed that indole conditioning of DCs robustly dampened their subsequent responses to TLR-mediated inflammatory stimuli. Thus, our hypothesis is that treating macrophages with indole will result in a similar suppression of pro-inflammatory responses.

Results

Indole pre-conditioning suppresses pro-inflammatory cytokine production in macrophages

We first investigated pro-inflammatory cytokine production in macrophages. Bone marrow-derived macrophages (BMDMs) were cultured to high purity (illustrated in Figure 3), routinely achieving >96% of cells bearing the hallmark macrophage signature of CD11b and F4/80 expressed on their surface. In our DC study, we typically conditioned DCs with indole during their development from bone marrow precursors in

order to mimic the proximity of DC progenitor cells to indole in the intestinal microenvironment during development. However based on the unique nature of intestinal macrophage ontogeny, we decided that a shorter treatment with indole would be more physiologically relevant as these cells are short-lived and continually being replenished from the circulation. We treated BMDMs for 18 hours with indole, followed by a 4 hour stimulation with either a very high (2 ug/ml) or low (250 ng/ml) dose of LPS. Intracellular cytokine staining revealed that production of both TNF and IL-12 were inhibited by nearly 2-fold in macrophages treated with 1 mM indole (Figure 49). To see if recently arrived macrophage precursors might also be affected, we next treated BMDMs for just 4 hours with indole, followed by a 2 hour stimulation with the same LPS concentrations. Interestingly, we observed a similar inhibition of both cytokines even with this shorter duration of indole conditioning (Figure 49). Based on this observation, we conclude that even a short treatment with indole is sufficient to modify macrophage functions in response to inflammatory stimuli.

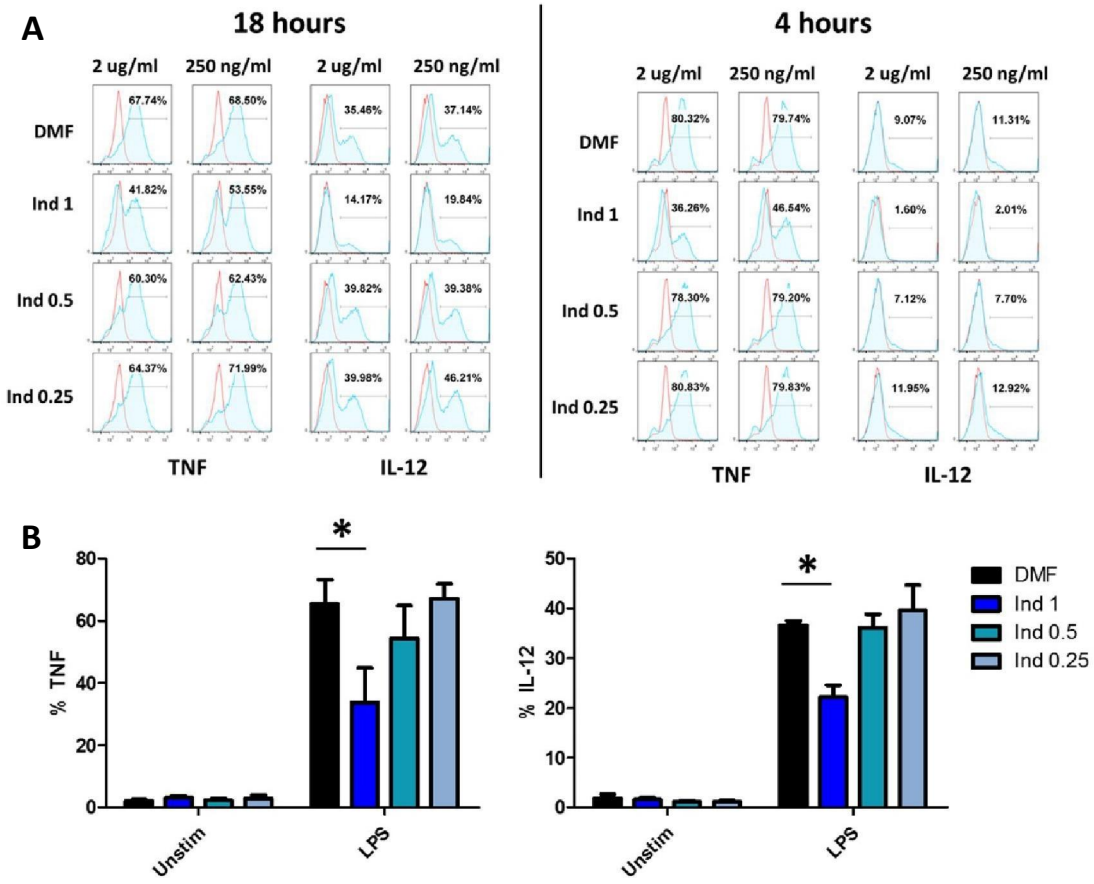

Figure 49. Treatment for 18 or 4 hours with indole inhibits TNF and IL-12 production in BMDMs.

BMDMs were treated with indole or DMF control for 18 hours or 4 hours, followed by stimulation with LPS (2 ug/ml or 250 ng/ml) for 4 hours in the presence of Golgiplug. BMDMs were then stained for intracellular TNF or IL-12 and analyzed by flow cytometry. (A) Red histograms represent unstimulated controls. Percentage of BMDMs producing cytokine are indicated on plots. (B) Aggregate data from 4 experiments with 18 hours indole treatment for TNF production (left panel) or IL-12 production (right panel). * $p < 0.05$ by Student's *t* test.

Macrophages and monocytes are the predominant source of harmful TNF production in inflammatory diseases (144), so we chose to test indole's effects on primary macrophages *ex vivo*. Bulk splenocyte cultures were treated with indole overnight, then stimulated with LPS or heat-killed *S. typhimurium* to induce cytokine production. Splenic macrophages were distinguished by flow cytometric analysis as those cells negative for lineage markers and expressing very high levels of the integrin CD11b, a population that has been demonstrated to be macrophages derived from conventional hematopoiesis (145). Here, indole conditioning inhibited TNF production by primary macrophages by an astonishing 75% compared to solvent controls (Figure 50). When contemplating this result, it is important to consider that these were heterogeneous cultures, thus indole was also in contact with many other immune cells. As such, one explanation could be that indole affected other cells in the culture which then modified macrophage function indirectly. To rule out this possibility, future studies with purified primary macrophages would be required. However given the profound effects of indole on purified BMDMs, we theorize that the effect observed is due to indole's direct action upon the splenic macrophages.

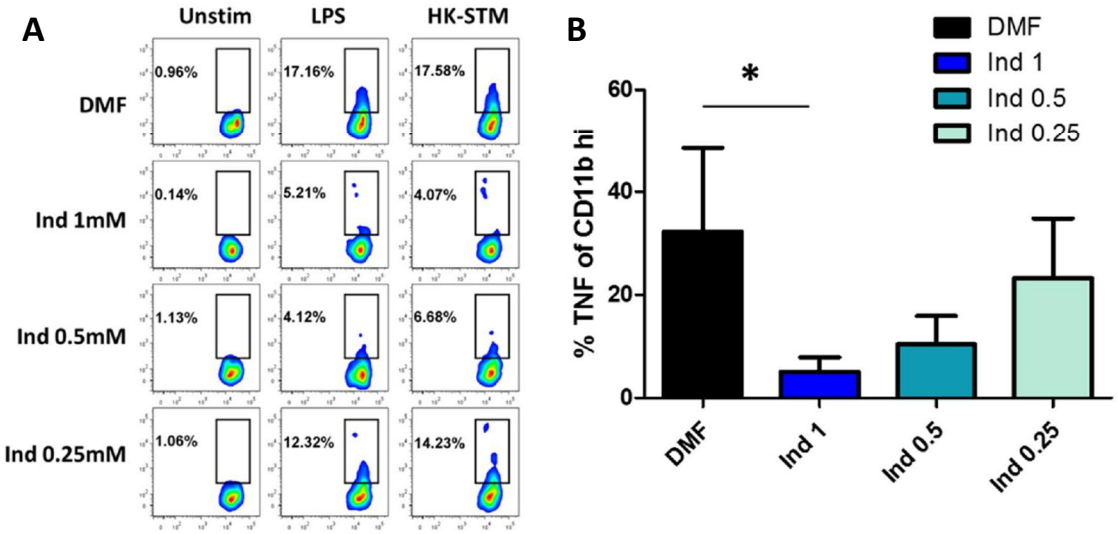

Figure 50. Indole conditioning inhibits TNF production in splenic macrophages *ex vivo*.

Splenocytes were cultured for 24 hours with indole or DMF control, then stimulated with LPS (10 ug/ml) or HK-STM (4 x 107 CFU) for 6 hours in the presence of Golgiplug. Cultures were then stained for CD11b and CD11c and intracellular TNF and analyzed by flow cytometry. Analysis was confined to the distinct CD11bhi CD11cneg macrophage population. (A) Representative plots with percentage of TNF producing macrophages indicated. (B) Mean of three independent experiments. * $p < 0.05$ by Student's *t* test.

Pro-inflammatory signaling pathways are inhibited by indole conditioning in macrophages

Based on our observation that cytokine production in macrophages is altered by a relatively short treatment with indole, we predicted that upstream pro-inflammatory signaling pathways are also affected. We focused our efforts on signaling pathways that we found to be inhibited by indole in DCs. The transcription factor, Stat3, is a central mediator of numerous physiological processes and has been suggested to be the predominant signal transducer downstream of gp130- like receptors (102). Contradictory findings have been reported for the role of Stat3 in APC signaling pathways, likely due to the pleiotropic nature of Stat3. Macrophage- specific ablation of Stat3 has been associated with dysregulation and heightened pro- inflammatory cytokine production in response to endotoxin (146); conversely, hyper- activation of Stat3 in a transgenic mouse model resulted in inflammation and macrophage-specific functional defects (147). These reports suggest that Stat3 activation is tightly regulated and that the macrophage lineage is sensitive to Stat3 activity.

Stimulation of BMDMs with the canonical Stat3 activator, IL-6, following overnight indole conditioning revealed a robust inhibition of Stat3 phosphorylation. Treatment with indole reduced the number of macrophages undergoing Stat3 phosphorylation by 50% and induced a similar inhibition of mean fluorescent intensity of the activated population (Figure 51). These data indicate that the Stat3 signaling pathway in macrophages is highly sensitive to indole.

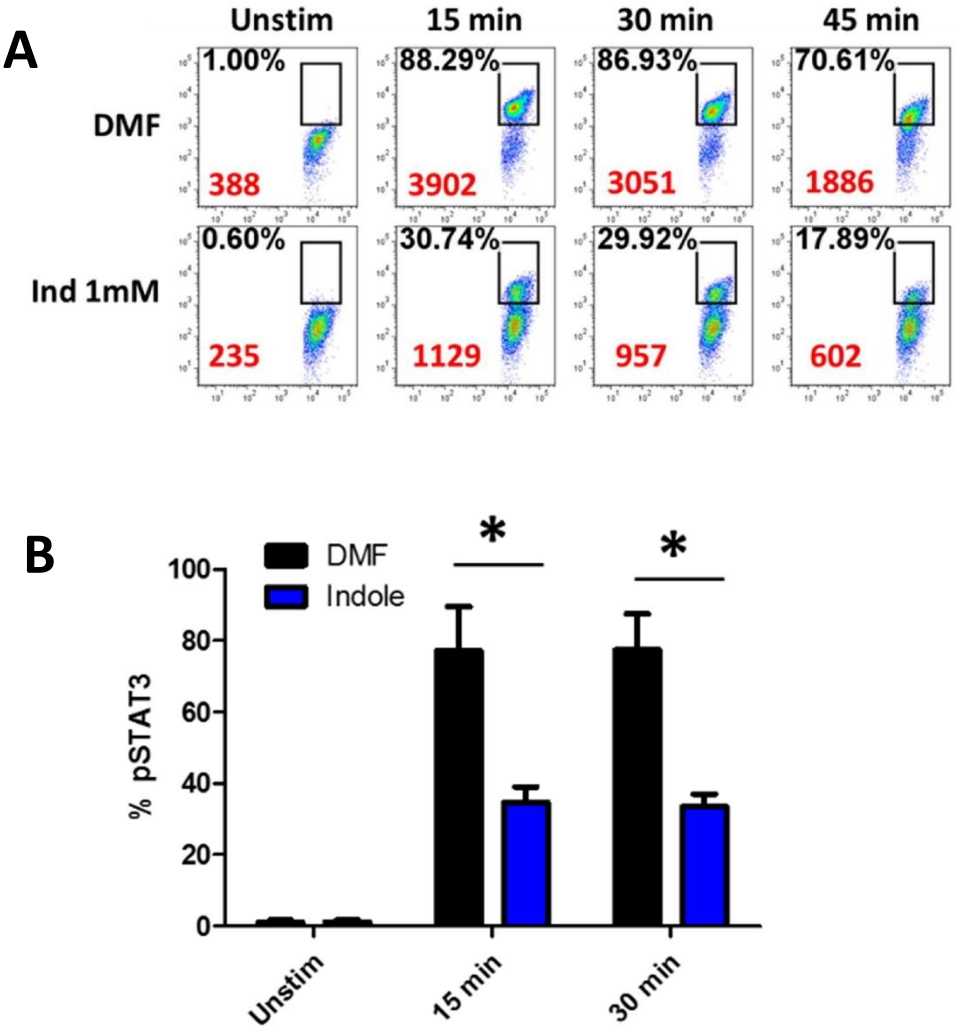

Figure 51. Indole inhibits IL-6-induced pStat3 signaling in BMDMs.

BMDMs were treated with indole or DMF control for 18 hours, then stimulated with IL-6 (10 ng/ml) for the given times. Cells were stained for phospho-Stat3 and analyzed by flow cytometry. (A) Representative plots indicating percentage of pStat3-positive BMDMs. MFI values for pStat3+ population indicated in red. (B) Mean of three independent experiments. * $p < 0.05$ by Student's t test.

The PI3K/Akt and MAPK/Erk signaling pathways regulate many cellular processes including survival, apoptosis, growth, and cytokine production. We have demonstrated in this study that Akt phosphorylation is inhibited by indole in DCs. Similarly, we found that indole conditioning dampened pAkt as well as pErk signaling in LPS and CpG-stimulated macrophages by approximately 50% (Figure 52). The inhibition of LPS-induced Akt activation has been demonstrated to decrease TNF production in macrophages (148). As such, indole's upstream inhibition of Akt activation might provide a mechanism for the observed suppression of TNF production. Further investigation of this pathway would be required to test this hypothesis. Akt suppression has also been associated with decreased nitric oxide production in macrophages (148). In a future study, it would be useful to determine whether indole also produces this effect as NO is an important inflammatory mediator in macrophages.

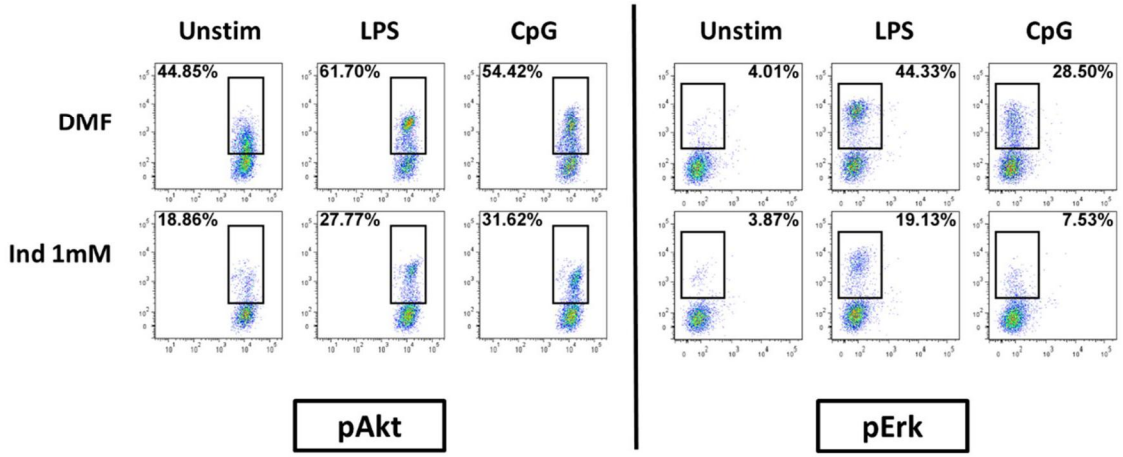

Figure 52. Indole inhibits pAkt and pErk signaling in BMDMs.

BMDMs were treated with indole (1 mM) or DMF control for 18 hours, then stimulated with LPS (10 ug/ml) or CpG (5 uM) for 30 minutes. Cells were stained for phospho-Akt (left panel) or phospho-Erk (right panel) and analyzed by flow cytometry. Percentage of positive-staining BMDMs indicated on plots. Representative data from one of three independent experiments.

It has been reported that specific inhibition of Erk in macrophages prior to LPS stimulation resulted in decreased NO production, while TNF and IL-12 expression were unaffected, indicating that Erk signaling is distinct from pro-inflammatory cytokine production (149). In support of this, butyrate conditioning of colonic lamina propria macrophages inhibited IL-6 and IL-12 expression, while MAP kinases including pErk were unaffected (70). In contrast, another group has reported short chain fatty acid-induced inhibition of Erk1/2 phosphorylation as well as decreased inflammatory mediators (iNOS, TNF, and IL-6) in the RAW264.7 macrophage line (150), suggesting potential crosstalk. These opposing findings demonstrate that MAP kinase signaling and pro-inflammatory cytokine production can be regulated independently. However, our observation that signaling pathways downstream of the LPS-detecting TLR4 are inhibited suggests that indole acts at the level of TLR signaling in its suppression of pro-inflammatory cytokine production.

Excessive production of inflammatory mediators in macrophages is associated with numerous inflammatory diseases, including Crohn's disease (151), metabolic disease (152), rheumatoid arthritis (153), and pulmonary fibrosis (154). Our preliminary observations suggest that indole is an effective small molecule inhibitor of multiple pro-inflammatory pathways and downstream cytokine responses. Future investigation of indole for anti-inflammatory therapeutic use might be useful in a number of clinically relevant diseases.

The effects of indole on macrophages appear to be AhR-independent

In the current study, we have observed that indole's effects in DCs are independent of the aryl hydrocarbon receptor, whereas concurrent work in our lab has revealed AhR dependence in indole's effects on CD4+ T helper cells (Steinmeyer et al, in preparation). To address the question of indole and AhR dependence in macrophages, we acquired AhR-deficient mice and generated BMDMs from these animals. In repeating our earlier experiments using BMDMs derived from AhR-knockout mice alongside wild-type littermate controls, we found nearly identical effects of indole regardless of AhR status on the production of pro-inflammatory cytokines TNF and IL-12 (Figure 53). In both cases, cytokine production was reduced by approximately 2-fold subsequent to conditioning of BMDMs with 1 mM indole.

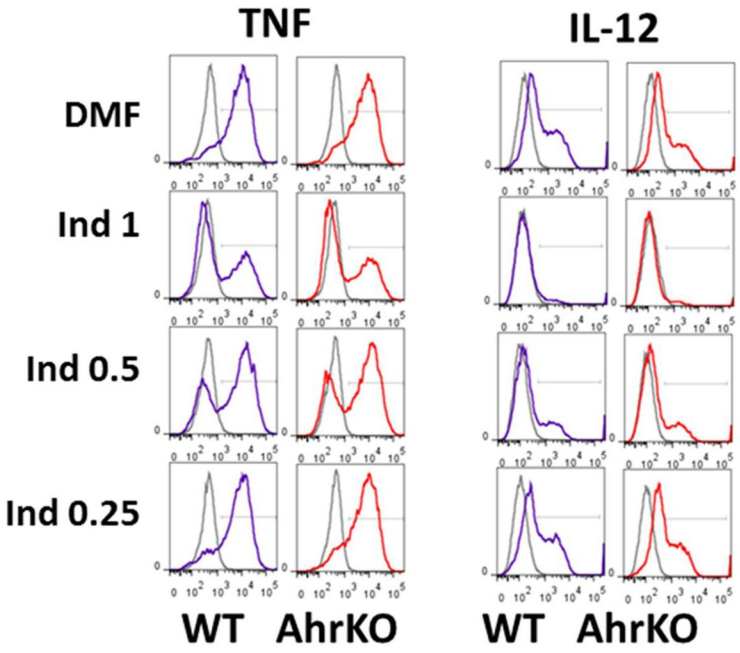

Figure 53. The indole-mediated inhibition of TNF production in BMDMs is AhR-independent.

Flow cytometric analysis of BMDMs generated from WT or AhR-KO mice and treated with indole or DMF control for 18 hours. BMDMs were stimulated for 4 hours with LPS (50 ng/ml) in the presence of Golgiplug, then stained for intracellular TNF (left panels) and IL-12 (right panels). Gray histograms represent unstimulated samples and colored histograms are LPS-stimulated. Representative data from one of three independent experiments.

Similarly, we repeated our *ex vivo* indole conditioning of splenocytes with tissues from AhR-deficient and wild-type mice and analyzed TNF production. Overall TNF production was reduced by 25% or more in AhR-deficient macrophages compared to wild-type controls. However, a marked inhibition of cytokine production was observed with indole treatment in both groups, indicating AhR independence (Figure 54). Importantly, in this set of experiments we stimulated splenocytes with LPS, heat-killed *S. typhimurium*, and CpG. Indole pre-conditioning dampened TNF production in response to all three stimuli, indicating that the observed effects are not LPS-restricted but can be achieved through the activation of different TLRs. Further, our investigation revealed that indole's robust inhibition of IL-6- mediated Stat3 phosphorylation in BMDMs was entirely AhR-independent (Figure 55). Conditioning with 1 mM indole reduced Stat3 signaling by over 60% regardless of AhR status, confirming that an alternative receptor is responsible for mediating indole's effects in APCs.

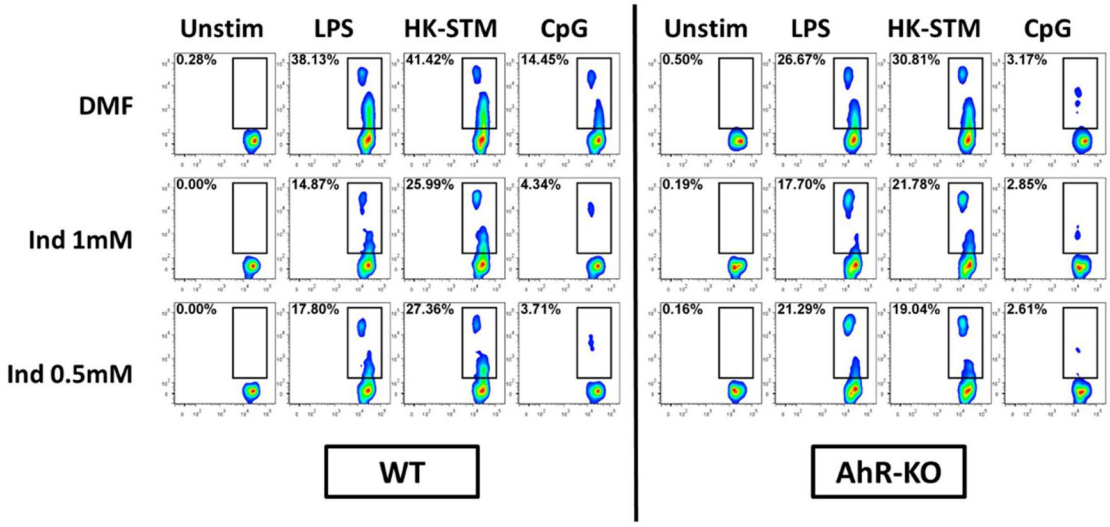

Figure 54. The indole-mediated inhibition of TNF production in splenic macrophages is AhR-independent.

Splenocytes from WT (left panel) or AhR-KO (right panel) mice were treated with indole or DMF control overnight, followed by stimulation for 6 hours with LPS (10 ug/ml), HK-STM (4 x 10^7 CFU), or CpG (3 uM) in the presence of Golgiplug. Cells were surface stained for CD11b and CD11c followed by intracellular staining for TNF. Flow cytometric analysis was confined to the distinct $CD11b^{hi}$ $CD11c^{neg}$ macrophage population. Percentage of TNF-positive macrophages indicated on plots. Representative data from one of three independent experiments.

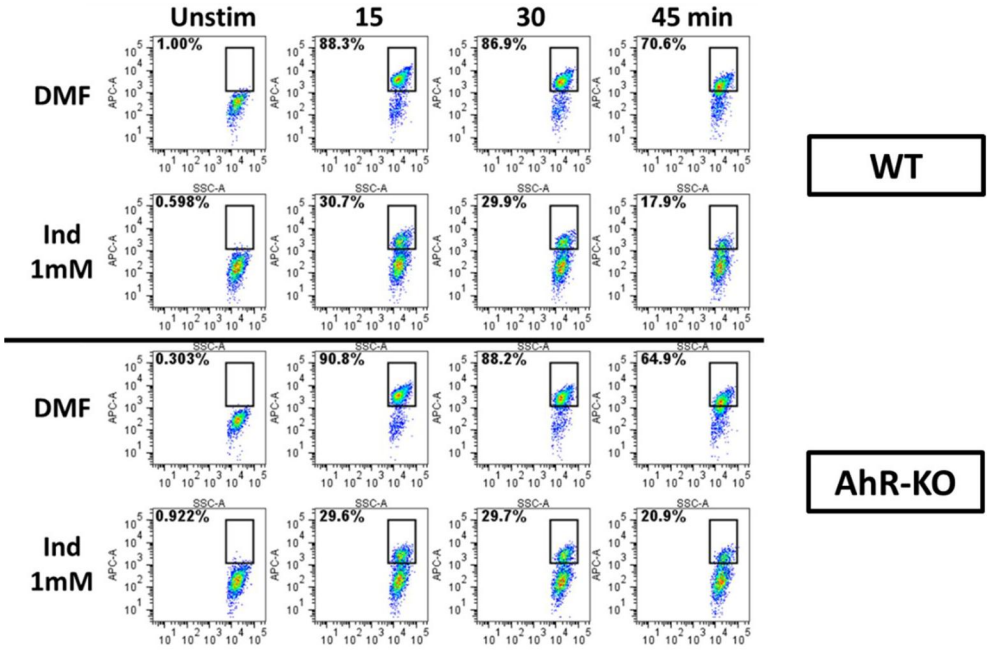

Figure 55. The indole-mediated inhibition of pStat3 signaling in BMDMs is AhR-independent.

BMDMs were generated from WT (upper panel) or AhR-KO (lower panel) mice and treated with indole (1 mM) or DMF control for 18 hours. BMDMs were then stimulated with IL-6 (10 ng/ml) for the given times and stained for phospho-Stat3, which was detected by flow cytometry. Percentage of pStat3-positive BMDMs indicated on plots. Representative data from one of three independent experiments.

Discussion

While the macrophage portion of our study is not to be considered comprehensive, we have successfully demonstrated that the microbiota metabolite, indole, induces profound alterations in macrophage signaling and pro-inflammatory cytokine production. These observations are important for multiple reasons. First, they reveal that indole's effects are common to multiple antigen-presenting cell subsets and not restricted to DCs. This information helps to guide our search for the mechanism that mediates the effects of indole: based on similar observations in DCs and macrophages, a logical approach would be to focus on pursuing receptors that are present in both cell types. We could test receptor candidates using knock-out animal models or by incorporating receptor-blocking antibodies into our study to determine whether the effects of indole are reliant upon a particular cellular receptor.

Second, they indicate that even a relatively short period of contact with indole is sufficient to induce physiological changes. This suggests that monocytes entering the intestines from circulation likely have the opportunity to be "instructed" by indole to dampen their inflammatory responses to TLR ligands, thus contributing to the tolerogenic landscape of the intestinal environment. This is important because compared to other tissue-resident macrophages, intestinal macrophages have a distinctly short half-life and are under constant renewal (116) and therefore sensitivity to local conditioning factors is likely a key factor in determining their function. As the most abundant mononuclear phagocytes in the lamina propria (155), macrophages play a key role in

promoting a tolerogenic environment and identifying a previously unknown endogenous signal that potentially drives this function would be novel. It has been demonstrated that during colitis, monocytes arriving in the intestines respond to pattern recognition receptor ligands by differentiating into pro-inflammatory macrophages that produce TNF and IL-12 among other inflammatory mediators (156). Because indole levels are low during colitis (93), we believe this supports our hypothesis that under steady-state conditions, high levels of indole in the intestines educate antigen-presenting cells to dampen pro-inflammatory responses to TLR stimuli. Under conditions of intestinal inflammation, however, indole levels are significantly depleted and macrophage precursors entering the gut are not exposed to this tolerogenic signal and instead further exacerbate inflammation as a result.

Previously established gut-centric factors which specifically modify macrophage function are few. One such identified factor is intestinal epithelial cells, which produce tolerance-inducing molecules such as thymic stromal lymphopoeitin (TSLP), TGF-β, semaphorin 7A, and prostaglandin E2 to which intestinal macrophages are responsive (157). The reported HDAC-inhibitory activity of the SCFA, butyrate, was a seminal finding in the pursuit of microbiota components that confer tolerogenic properties upon intestinal macrophages (70). Here, the current study provides new information as to a novel microbiota signal which directly suppresses pro-inflammatory responses in macrophages. Both primary cultured and splenic macrophages were highly sensitive to indole upon a relatively short treatment period, supporting our hypothesis that indole is a

biologically relevant conditioning factor which suppresses inflammatory responses in macrophages upon arrival in the intestinal microenvironment. Further studies will be directed towards identifying the receptor for indole in APCs as well as investigating the effects of indole on macrophage-focused properties, such as bacterial clearance and epithelial wound healing. Based on our current and previous work that identifies indole as a beneficial signal in the gut, we hypothesize that indole will promote pathogen clearance and wound healing in intestinal macrophages.

CHAPTER V

METHODS

Mice

C57BL/6 (WT), B6.Cg-Tg(TcraTcrb)^{425}Cbn/J (OT-II), and B6.PL-Thy1^{a}/CyJ (B6-Thy1.1) mice were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL/6-Ahr$^{tm1.2Arte}$ (AhR-KO) mice for initial experiments were a kind gift from Dr. Bhagavatula Moorthy (Baylor College of Medicine, Houston TX). Further AhR-KO mice were purchased from Taconic Farms (Rockville, MD). Mice were maintained in a specific pathogen-free animal facility located at Texas A&M University Health Science Center. All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines under an approved animal use protocol. Mice were age and gender-matched for each experiment and used between 6 and 13 weeks of age.

Reagents

Indole and all-trans retinoic acid were purchased from Sigma (St. Louis, MO). Indole was dissolved in dimethylformamide (DMF) and added to cultures at indicated concentrations. Recombinant murine GM-CSF and TGF-β1 were purchased from R&D Systems (Minneapolis, MN). Ultra-pure LPS from Invitrogen (San Diego, CA) was used for DC activation. Cell Rox Deep Red reagent for detection of reactive oxygen species (ROS) was obtained from Molecular Probes (Invitrogen, USA). Pacific Blue

Succinimidyl Ester (PBSE) was used for discrimination of viable cells (Life Technologies). $OVA_{323-339}$ peptide was purchased from AnaSpec, Inc. (Fremont, CA). Antibodies used for flow cytometry included CD4 (clone RM-5), CD11c (HL3), CD86 (GL1), I-Ab (AF6-120.1), TNF (MP6-XT22), IL12 (c15.6), IL-6 (MP5-20F3), phospho-Stat3 (4/P-STAT3) and LPAM-1/α4β7 (DATK32) purchased from BD Biosciences (San Jose, CA). Anti-Foxp3 (FJK-16s), CCR9 (CW-1.2), CD45R/B220 (RA3-6B2) and TLR4/MD-2 (MTS510) were purchased from eBioscience (San Diego, CA) and phospho-Akt (193H12) and phospho-NF-kB p65 (93H1) were purchased from Cell Signaling Technology (Beverly, MA).

Cell culture

DC culture and stimulation

The murine dendritic cell line DC2.4 was kindly provided by Dr. Kenneth Rock, University of Massachusetts Medical Center. DC2.4 cells were maintained in complete DMEM medium (Invitrogen, Grand Island, NY) supplemented with 5% fetal calf serum (FCS). All primary cells were cultured in RPMI 1640 supplemented with 2-mercaptoethanol, gentamicin, penicillin, streptomycin, and 10% FCS (all from Life Technologies). Bone marrow-derived dendritic cells were cultured as previously described (94) with slight modifications. Briefly, bone marrow cells were harvested from murine femurs and cultured in complete media supplemented with 10% FCS, 20ng/ml GM-CSF (Peprotech), and 10ng/ml IL-4 (Peprotech). Media was replenished at day 2, 4, and 6 of the 7 day culture. Indole, RA, or solvent control were added to the cultures at

day 3 where indicated. Stimulation of dendritic cells at the given concentrations of LPS or heat-killed *S. typhimurium* were performed for 4 hours (DC2.4 and BMDCs) or 6 hours (primary splenic DCs) in the presence of Golgi-Plug protein transport inhibitor (BD Biosciences) to measure cytokine production, or for 24 hours (co-stimulatory markers).

Macrophage culture

Bone marrow was harvested as described above and cultured at a density of 1 x 10^6 cells in 10 ml complete media supplemented with 10 ng/ml M-CSF (Peprotech) in 100mm x 15mm petri dishes. Media with cytokine was replenished on day 3. Adherent and semi-adherent cells were recovered on day 7, plated, and treated with indole prior to downstream experiments.

Assays

NF-kB activity assay

The DC2.4-NFkB-GLuc cell line was generated by our collaborators (Arul Jayaraman Lab, Chemical Engineering Dept, TAMU). A reporter plasmid with tandem repeats of NF-kB response element was designed and integrated into the genomic DNA of the DC2.4 cell line via lentiviral transduction. A minimal inducible cytomegalovirus (CMV) promoter was cloned upstream of GLuc (*Gaussia* luciferase, a secreted reporter) to monitor NF-kB activation. The inducible promotor is inactive in the absence of NF-kB binding to DNA. DC2.4-NFkB-GLuc cells were pre-treated with indole and/or TGF-β

for 24 hours followed by LPS stimulation (10 ug/ml). Culture supernatants were collected at 8 and 24 hours and assayed for GLuc using Biolux Gaussia Luciferase assay kit (New England BioLabs).

Cytotoxicity assay

The CytoTox96 non-radioactive cytotoxicity assay (Promega) was used to identify cytotoxic effects of indole. Cells were treated with or without indole for various durations and the amount of lactate dehydrogenase (LDH) released into the culture supernatant was measured.

Reactive oxygen species (ROS) production

Reactive oxygen species production was measured using the CellRox Deep Red Reagent (ThermoFisher). Briefly, cells were treated with or without indole for 18 hours, then labeled with CellRox (5 uM) for 30 minutes and washed with PBS followed by LPS stimulation. Presence of ROS oxidizes the CellRox reagent to a fluorescent state that can be subsequently analyzed via flow cytometry.

Nitric oxide (NO) production

Nitric oxide concentration was determined by measuring nitrite levels in culture supernatants via the Griess reaction (178). Briefly, cells in the presence or absence of indole were stimulated with LPS for 24 hours. Supernatants were then incubated with Griess reagent for 10 minutes and then absorbance was measured at 550nm.

Antigen-uptake assay

Ovalbumin (OVA)- Alexafluor 488 conjugate (Molecular Probes) was used for measuring antigen uptake by DCs. Subsequent to 24 hour indole conditioning, cells were incubated with 1µg/ml OVA-A488 at 4°C or 37°C for 1 or 4 hours. Cells were then washed 3 times with ice-cold PBS containing 0.5% BSA to terminate uptake. Cells were surface stained for CD11c and fixed with 4% paraformaldehyde. Uptake of OVA-A488 was measured by flow cytometry.

Aldehyde dehydrogenase activity

Activity of aldehyde dehydrogenase (ALDH) in individual BMDCs was measured using an Aldefluor staining kit (Stem Cell Technologies) according to the manufacturer's instructions with minor modifications. Briefly, BMDCs were incubated with Aldefluor substrate (150 nm) for 45 minutes at 37°C, with or without ALDH inhibitor 4-(diethylamino) benzaldehyde (DEAB) at (100 µm). Subsequently, cells were stained with CD11c in cold Aldefluor assay buffer and then analyzed by flow cytometry.

Real-time PCR

Total RNA was extracted from BMDCs using RNeasy Mini Kit (Qiagen) and then retro-transcribed using cDNA Superscript Mastermix (Quanta). Quantitative PCR reactions were carried out in triplicate using Perfecta SYBR Green Fast Mix, Low ROX (Quanta). Gene expression levels for each individual sample were normalized to 18s. Mean relative gene expression was determined and differences were calculated using the

$2^{-\Delta C(t)}$ method. The following primer sequences (5'-3') were used:

Murine aldh1a2

F: CATGGTATCCTCCGCAATG **R**: GCGCATTTAAGGCATTGTAAC

Murine 18s

F: TCAACTTTCGATGGTAGTCGCCGT **R**: TCCTTGGATGTGGTAGCCGTTTCT

ELISA

Cytokine concentration in the supernatant of BMDC cultures was assessed using commercially available ELISA kits for IL-10 (Peprotech) and TGF-β (eBioscience) per the manufacturer's instructions.

Chemotaxis assay

24-well Transwell cell culture plates with 8.0 uM polycarbonate membranes were used for this assay (Corning) along with chemokines CCL25 (CCR9 ligand), CCL19 and CCL21 (CCR7 ligands; all from Peprotech). A volume of 600 ul cell culture media containing 1 ug/ml of either CCL25 or CCL19 and CCL21 was placed in the bottom chamber, followed by the transwell insert. BMDCs conditioned with indole, RA, or DMF from day 3 were harvested and washed on day 7. Exactly 100 ul of cell culture media containing 4.5 x 10^5 BMDCs was added to the upper chamber of the transwell. Controls without the presence of chemoattractants were included. Plates were incubated for 3 hours at 37°C, at which point transwell inserts were removed and migrated cells in

the bottom chamber were collected and counted. Migration is expressed as a percentage of the initial cell number.

DC-T cell co-cultures (polyclonal stimulation)

Naïve CD4+CD25- T cells from pooled spleen and lymph nodes of C57BL/6 wild-type mice were sorted to high purity (>97%) on a BD FACS Aria II flow cytometer (BD Biosciences). Antibodies used for staining were αCD4 (GK1.5) and αCD25 (PC61.5). BMDCs were previously treated with indole from day 3. On day 7, DCs were washed extensively prior to being co-cultured with the naïve FACS-sorted T cells for 72 hours in a 96-well round-bottom tissue culture plate in the presence of soluble anti-CD3 at 5ug/ml (BioXcell clone 145-2C11) as well as 2 ng/ml TGF-β (Peprotech) and/or 100 U/ml IL-2 (Roche). Subsequently, cells were stained for flow cytometric analysis using αCD4 (GK1.5) and αFOXP3 (FJK-16s).

DC-T cell co-cultures (monoclonal stimulation)

Naïve CD4+CD25- T cells of spleen and lymph nodes from OT-II mice were isolated by negative selection via column purification using the Naïve CD4+ T Cell Isolation Kit (Miltenyi Biotec). Naïve CD4+ T cells (>96% pure) were labeled with Cell Proliferation Dye efluor450 (eBioscience) following manufacturer's instructions then co-cultured with OVA peptide-loaded BMDCs at a ratio of 2:1 for 72 hours in a 96-well round-bottom tissue culture plate. BMDCs were previously treated with indole or RA from day 3. On day 7, DCs were washed extensively prior to being loaded with $OVA_{323-339}$ peptide

(10ug/ml) for 1 hour at 37°C. BMDCs were then washed three times and plated with naïve CD4+ T cells. RA or TGFβ were added to co-cultures as indicated. Following 3 days of culture, cells were stained with antibodies to CD4, CCR9, and LPAM-1 (α4β7). Intracellular staining for Foxp3 was performed using a Foxp3 staining kit (eBioscience). The remaining CD4+ helper T cell lineages Th1, Th2, and Th17 were identified by their cytokine production. For Th2 and Th17 differentiation cultures, lineage skewing cytokines were included for the duration of the co-culture. For Th2 skew, cultures received 0, 10, 50, or 100 ug/ml of each αIFN-γ (BioXCell R4-6A2) and IL-4 (Peprotech). For Th17 skew, cultures received 0, 10, or 50 ng/ml each of TGF-β (Peprotech), IL-6 (Peprotech), and IL-23 (R&D). Production of cytokines was stimulated by the addition of PMA and ionomycin (Sigma-Aldrich) during the last five hours of culture. Cytokines were retained inside the cells by the addition of the protein transport inhibitor, Golgiplug (BD) at the time of stimulation. Cells were fixed and permeabilized using the Foxp3 staining kit (eBioscience). Antibodies used for intracellular cytokines were αIFN-γ-PE (eBioscience XMG1.2), αIL-4-FITC (eBioscience BVD6-24G2), and αIL-17-efluor660 (eBioscience eBio17B7) to detect Th1, Th2, and Th17 cells, respectively.

DC-T cell co-cultures without cell contact

Naïve CD4+CD25- T cells were FACS-sorted and placed in the bottom of a 24-well Transwell cell culture plate with 0.4 uM polycarbonate membranes (Corning) that had been coated with 5 μg/mL αCD3 (BioXcell 145-2C11) and 2 μg/mL αCD28 (BioXcell

37.51). TGF-β was added at the indicated concentrations. The insert was placed in the well and washed, Day 3-treated BMDCs were added to the top chamber such that culture media was shared, but the cell types were kept physically separate. The T cell to DC ratio was 2:1, as in other co-culture experiments.

T cell activation in the presence of BMDC supernatant

Naïve CD4+CD25- T cells were FACS-sorted as described above and plated in 96-well, αCD3 and αCD28-coated tissue culture plates in a volume of 100 ul cell culture media. An equal volume of spent BMDC supernatant was added, in the presence or absence of exogenous TGF-β, and T cells were cultured for 72 hours. To generate supernatants, day 3-treated BMDCs were collected on day 7, washed extensively and plated in a 48-well cell culture plate at 5 x 10^5 cells per well in 500ul fresh complete media for 24 hours in the presence or absence of 1 ug/ml LPS. Plates were then centrifuged and supernatants were collected, filtered, and stored at -20°C until use.

In vivo *T cell activation by indole-treated BMDCs*

Naïve CD4+CD25- T cells from spleens and lymph nodes of OT-II mice were isolated by column purification as described above and resuspended in sterile Hanks Balanced Saline Solution (HBSS). Approximately 2 million T cells were transferred via i.v. injection (retro-orbital route) into recipient B6-Thy1.1 mice. Day 7 BMDCs cultured with indole, RA, or DMF from day 3 were washed and loaded with OVA peptide as described above. Two million BMDCs were resuspended in sterile HBSS and transferred

i.p. into the same B6-Thy1.1 recipient mice 5 hours subsequent to T cell transfer. After 5 days, tissues were harvested for cellularity analysis as well as *ex vivo* stimulation for cytokine production.

BMDC transfer with DSS-colitis

BMDCs were cultured with indole, RA, or DMF from day 3 as described above. BMDCs were washed, counted, and resuspended in sterile PBS (Ca-Mg-) for transfer to WT C57BL/6 recipients. Two million BMDCs were transferred via i.p. injection on days 0, 6, and 9 of the experiment. Mice received 5% dextran sulfate sodium (DSS, MW= 36,000-50,000; MP Biomedicals) *ad libitum* in their drinking water from day 7 through day 14. Mice were weighed daily to monitor weight loss. Mice were euthanized on day 20 for subsequent tissue harvest and processing.

Statistical analysis

Data were analyzed using GraphPad Prism Software 5.01. Comparisons between DMF and indole were performed using a Student's *t* test. One-way ANOVA followed by Dunnett's post test was applied to compare differences in experiments with DMF, indole, and RA treatment groups. *P* values of less than 0.05 were considered to be statistically significant.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Subsets of dendritic cells in the GALT can produce retinoic acid from vitamin A and subsequently regulate immunity via mechanisms including imprinting lymphocytes with gut-homing specificity and driving Foxp3+ regulatory T cell development. Indeed, retinoic acid has been previously identified as an essential co-factor in the induction of Foxp3+ Tregs by bona-fide, CD103+ mucosal dendritic cells (55). Our overall findings reveal that a novel microbiota-derived metabolite, indole, conditions DCs towards a mucosal phenotype in a manner unique from retinoic acid and these indole-conditioned DCs are capable of promoting a regulatory phenotype in naïve T cells (Figure 56).

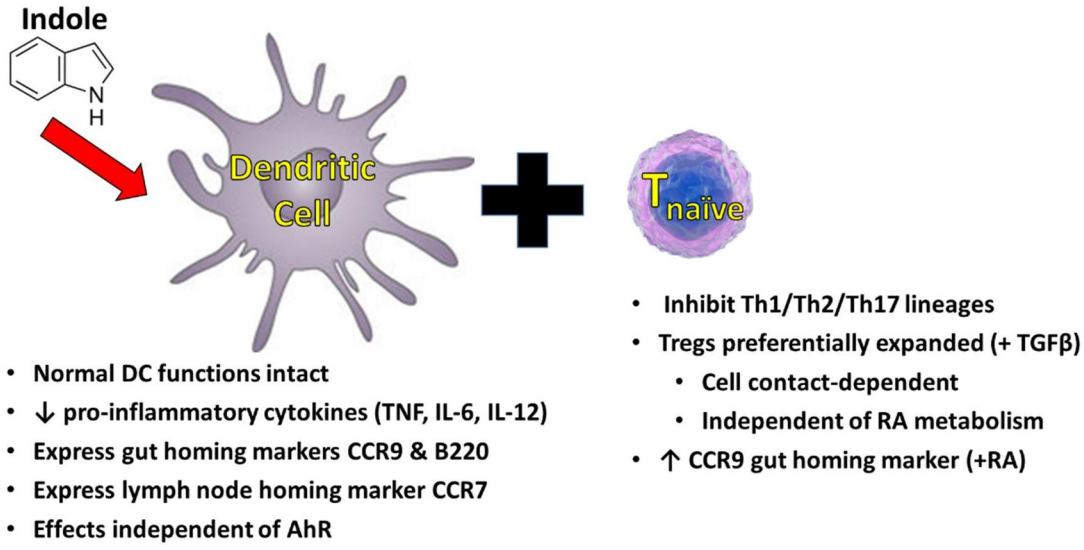

Figure 56. Indole conditioning promotes a tolerogenic phenotype and function in DCs.

The current study identifies numerous effects of indole conditioning on DCs as indicated in diagram. These properties are consistent with properties of bona fide mucosal DCs present in the intestinal environment.

The molecular mechanism for indole's effects on APCs remains unknown

While our study has revealed striking and previously unappreciated biology, the molecular mechanism of action by which indole affects APCs remains to be characterized. Despite our identification of indole as a ligand of the aryl hydrocarbon receptor (83), our work using AhR-deficient animals has revealed that indole's effects on APCs are entirely AhR-independent. The small size and freely diffusible nature of indole might suggest an intracellular receptor in APCs. In addition, indole seems to affect APCs in a manner similar to TGF-β and retinoic acid, both of which bind intracellular receptors. Further, indole's effects on APCs occur fairly rapidly, as we observed defects in phospho-signaling with as little as four hours indole pre-conditioning (data not shown).

Our observation that soluble factors do not appear responsible for indole-DC mediated Treg skew was surprising, as the comparable canonical factors RA and TGF-β are both soluble factors. However, the results of our study suggest that cell contact between the DC and naïve T cell is required for T cell lineage skewing and has thus directed our search for mechanism towards targets on the cellular surface. We have identified the programmed death (PD) 1-PD-ligand (PD-L) pathway as a possible candidate. PD-1 is an inhibitory receptor of the CD28/CTLA4 family expressed on activated lymphocytes and with a role in regulating tolerance and autoimmunity. Its ligands, B7-family members PD-L1 (B7H1) and PD-L2 (B7-DC), are expressed broadly on hematopoietic cells and restricted to DCs and macrophages, respectively (158, 159). It has been

demonstrated that PDL-1 can promote and support Tregs by inducing and enhancing Foxp3 expression in peripherally activated T cells (160). Further, this effect is mediated through the inhibition of Akt/mTOR signaling (160), an effect we have observed here in indole-treated APCs. Thus, indole-induced upregulation of PD-1 ligands on DCs might be a cellular mechanism by which indole-conditioned DCs promote Tregs, and we plan to test this hypothesis in subsequent mechanistic studies.

An intriguing finding in this study was that indole's direct effects on DC phenotype and function occurred in the absence of any exogenous co-factors, whereas indole-conditioned DCs required TGF-β or retinoic acid to affect naïve T cell activation. This might suggest that more than one receptor or pathway mediates indole's effects on APCs. Determining the mechanism(s) of action of indole on DCs is a crucial next step in this study. A transcriptomics approach would be useful to identify rational targets and signaling pathways that mediate the observed effects.

AKT/mTOR pathway as a potential target of indole in APCs

One pathway that we predict might mediate indole's effects on APCs is mammalian target of rapamycin (mTOR)/autophagy signaling. First described in 1991, TOR was identified as a target of the bacteria-derived immunosuppressant, rapamycin (161). mTOR is an evolutionarily conserved protein kinase that plays a central role in balancing cell growth and the lysosomal degradation process known as autophagy. As such, mTOR allows cells to adjust their metabolism based on environmental signals including nutrient

starvation and stress. Under low nutrient conditions, mTOR induces autophagy, which degrades intracellular components including damaged or non-essential organelles, misfolded proteins, and intracellular pathogens, thus providing additional energy sources to promote cell survival.

As demonstrated in chapter two, we have found that indole strongly inhibits Akt signaling in DCs. Hyperactivation of Akt is a hallmark of many solid tumors (162) and has been linked to tumor aggressiveness (163). While model systems have shown these tumors to be highly sensitive to mTOR inhibitors, use of the inhibitors in human cancer patients has shown minimal success due to the release of an inhibitory mechanism ultimately resulting in upregulated Akt and mTOR (164). It has therefore been suggested that small molecule mTOR inhibitors that prevent Akt activation might be highly useful as anticancer therapies. Based on our preliminary data, we would recommend that indole and related metabolites be investigated for development as potential therapeutics against tumors.

Our preliminary experiments suggest that, like rapamycin, indole induces autophagy in DCs (data not shown). The importance of autophagy in DCs has been well documented. For example the bacterial sensor, NOD2, has been shown to induce autophagy in DCs, an effect that was necessary for bacterial clearance as well as MHC-II-mediated antigen-specific CD4+ T cell responses in DCs (165). Thus, we predict that indole may provide a beneficial effect on host cell survival and intracellular bacterial clearance by regulating

the mTOR/autophagy pathway. Futures studies exploring effects of indole at multiple checkpoints along this pathway will be useful for clarification.

We have observed similar effects of indole and rapamycin on the inhibition of downstream mTOR target, phospho-S6 in naïve T cells (Figure 57), suggesting that both inhibit mTOR activity. This might indicate that indole acts as a "rapalog", mimicking rapamycin activity. However, we have found differential effects on DC cytokine production: indole inhibits pro-inflammatory IL-12 and does not affect anti-inflammatory IL-10, whereas rapamycin stimulates IL-12 production and inhibits IL-10 under similar LPS stimulation conditions (166). Thus, despite some apparent overlapping biological effects, indole cannot be characterized as a rapalog in DCs. It is interesting to note that while both bacteria-derived compounds, indole is a naturally-occurring microbiota metabolite present in the human GI tract whereas rapamycin is produced by the soil-dwelling bacterium, *Streptomyces hygroscopicus* (167) and thus bears no direct relevance to human physiology. Subsequent research efforts in our lab will be devoted to exploring this pathway as a potential target of indole in immune cells.

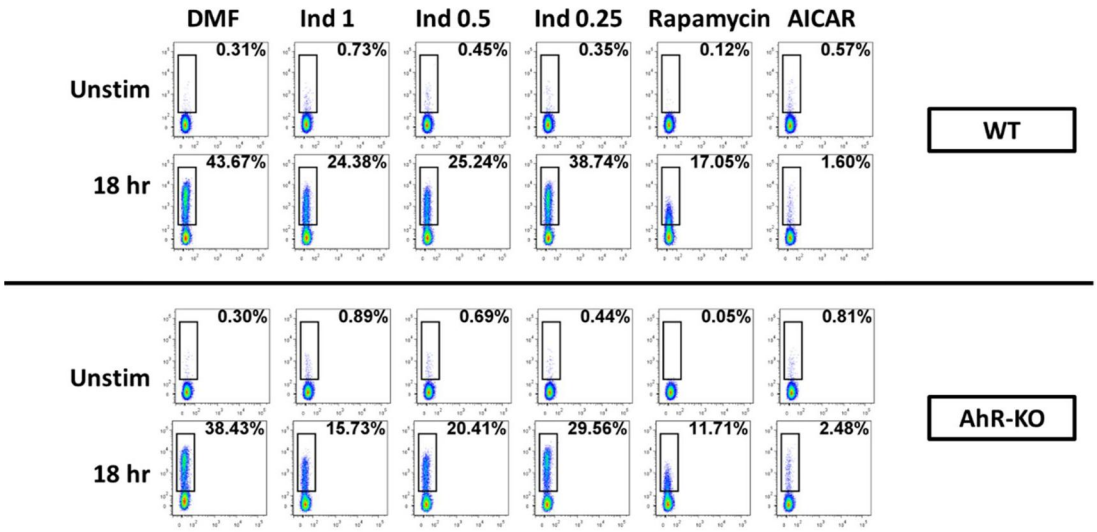

Figure 57. Indole inhibits phospho-S6 in purified CD4+CD25- T cells in an AhR-independent manner.

Wild type (upper panel) or AhR-KO (lower panel) CD4+CD25- T cells were sorted to high purity and cultured with indicated treatments for 18 hours. Treatment concentrations were indole at 1, 0.5, and 0.25 mM, Rapamycin at 25 nM, and AICAR at 0.5 mM. Phospho-staining was then performed for the S6 ribosomal protein and analyzed by flow cytometry. Percentage of pS6-positive cells indicated on plots. Representative data from one of two experiments.

Expand study to include macrophage-specific and B cell biology

This work largely focused on dendritic cells, as these antigen-presenting cells act as the predominant activators of naïve T cells *in vivo* and thus have a crucial role in bridging innate and adaptive immunity. However, macrophages are not only capable of activating naïve T cells, but they play an important role in preventing aberrant mucosal inflammation. Our abbreviated study indicated that indole affects macrophages in a very similar manner to DCs, and these effects appear AhR-independent in both immune cell subsets. Further work with indole focused on biology pertinent to macrophage functions, such as bacterial clearance and wound healing, would be enlightening.

While antigen presentation is not their primary role, B lymphocytes represent the remaining subset of professional antigen-presenting cells in addition to DCs and macrophages. B cells can engulf protein antigens and present the peptide: MHC-II complex to CD4+ T cells within lymphoid organs. The engaged helper T cell responds to this interaction by upregulating CD40 ligand and secreting cytokines, both signals that reciprocally activate the antigen-presenting B cell. The B cell will then proliferate and differentiate to an antibody-secreting plasma cell. In mucosal tissues, IgA is the dominant class of antibody and is produced locally by plasma cells in the mucosal wall (168). Secreted IgA binds the mucus layer covering the intestinal epithelium and serves a crucial protective role in the intestinal microenvironment by limiting the access of pathogens to mucosal surfaces, a phenomenon termed immune exclusion (169). In addition, IgA possesses minimal capacity to activate the classical complement pathway

or to act as an opsonin, therefore IgA does not induce inflammation and contributes to mucosal tolerance (170). Naïve B cell precursors of IgA-secreting plasma cells become activated in gut-associated lymphoid tissues, thus we predict direct contact of these precursor cells with indole or indole-conditioned DCs. We have demonstrated that primary splenic B cells are sensitive to treatment with indole, exhibiting similar inhibition in signaling as observed in DCs. We have found that 1 mM indole suppresses pAkt and pErk signaling by 50% or more in splenic B cells in an AhR-independent manner (Figure 58). Based on our observations thus far that indole promotes tolerance in several immune cell types, we hypothesize that indole may additionally contribute to immune homeostasis by stimulating B cell class-switching to IgA-producing plasma cells.

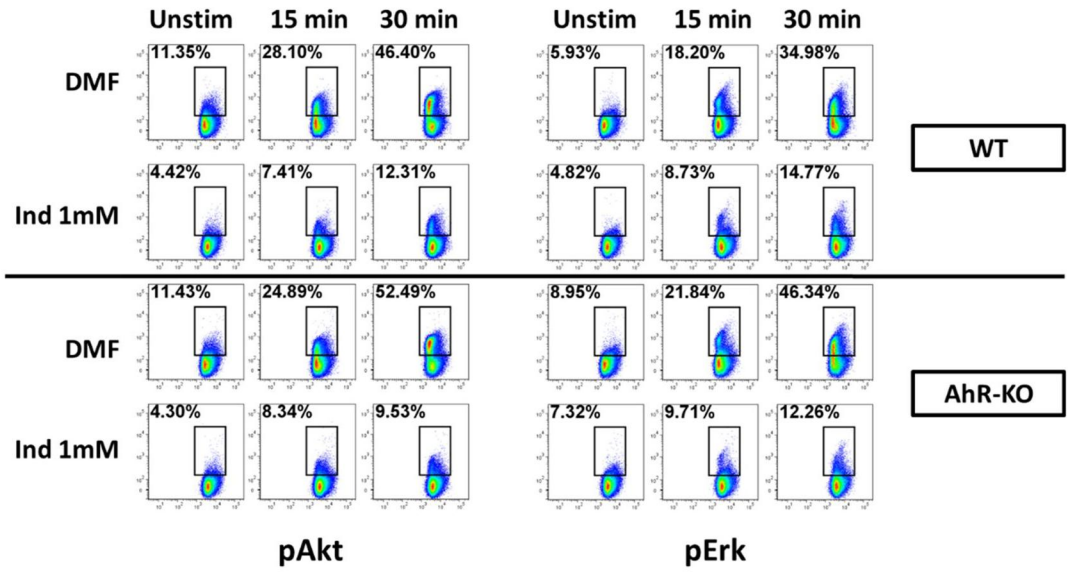

Figure 58. Indole inhibits pAkt and pErk signaling in splenic B cells in an AhR-independent manner.

Splenocytes from wild type (upper panel) or AhR-KO (lower panel) mice were cultured for 18 hours with indole or DMF control, followed by stimulation with CpG (3 uM) and phospho-staining for phospho-Akt (left) and phospho-Erk (right). Flow cytometric analysis was confined to B220+ B cells. Percentage of positive-staining B cells indicated on plots. Representative data from one of two experiments.

Continue pursuing *in vivo* protection in colitis models

In this study we have utilized the DSS murine model of colitis to test for *in vivo* protective effects of indole-conditioned DCs. While we did not observe protection, we believe that a number of factors might account for this result, including the number of DCs transferred and its timing, as well as severity of the inflammatory insult and a potential requirement for IL-6 as a reparative factor (137). In addition, route of indole administration is an important factor to consider as different routes likely have differing bioavailabilities and final concentrations in tissues. We have plans to continue pursuing this model with a modified approach, utilizing multiple cycles of low-dose DSS in order to more accurately represent the chronic colonic inflammation associated with inflammatory bowel disease in humans. Our prediction is that longer term conditioning of the mucosal tissues with adoptively transferred indole-DCs will reveal a protective effect by dampening the inflammatory signaling associated with DSS-colitis.

Murine models of intestinal inflammation are typically categorized as being induced by chemicals, gene knockout, or CD4+ T cell reconstitution of immune-deficient mice. A careful consideration of the available models and their mode of action is necessary to choose the most appropriate system. We chose the chemically induced DSS model due to its widespread use, reproducibility, and mechanism of intestinal injury. Oral delivery of DSS results in pathology analogous to human ulcerative colitis, as evidenced by epithelial layer damage and permeability, acute inflammation, and infiltration of neutrophils and macrophages (171). The other widely used chemically induced model is

administration of the haptenating agent, TNBS. This compound is administered intra-rectally and elicits a severe inflammatory response followed by tissue necrosis, resembling human Crohn's disease (172). Successful protection against colitis has been recently achieved in this model when DCs were treated with a microbiota component, then adoptively transferred to recipient mice prior to TNBS administration (73, 127). Based on this observation, it is possible that DCs play a more predominant role in the TNBS colitis model and as such, it may be worth considering the application of this model to our indole-conditioned DC system.

Examine potential benefit of indole in sepsis model

An alternative health condition that might benefit from indole-conditioned DC transfer would be sepsis. Each year over a million Americans suffer from septic shock, a life-threatening inflammatory response to overwhelming infection. The Agency for Healthcare Research and Quality lists sepsis as the most expensive condition treated in U.S. hospitals, costing more than $20 billion in 2011 and increasing on average annually by 11.9 percent (173). Despite decades of clinical trials targeting the treatment of sepsis, no significant therapeutic success has been achieved and the number of sepsis cases per year continues to rise. Novel treatment strategies are desperately needed. Lipopolysaccharide is a component of the cell wall of bacteria and known to be one of the primary inducers of septic shock. Sepsis is characterized by massive pro-inflammatory cytokine release and excessive recruitment of immune cells, which can lead to multiple organ failure, dangerously low blood pressure, and ultimately, death.

A review of the literature reveals the LPS sepsis model as a well-characterized, relevant and practical means of studying septic shock-induced death. Injecting rodents with LPS is the most extensively studied animal model of sepsis, utilized for over 40 years (174). This endotoxin model is consistent, reproducible, and easily standardized. Upon injection of LPS, a systemic inflammatory response is initiated that resembles the systemic inflammatory response in human patients with severe sepsis, characterized by increased circulating concentrations of numerous cytokines (175-177).

We have demonstrated that indole conditioning drastically dampens pro-inflammatory cytokine production by APCs upon LPS stimulation. Based on these observations, we propose that pre-treatment with indole will condition host cells to be less responsive to subsequent inflammatory insult, thus preventing death from septic shock. If successful, the further development of indole and other metabolites for therapeutic use in the context of preventing sepsis would be highly advantageous in a clinical setting and has the potential to save thousands of lives.

Additional microbiota metabolites as immune modulators

It is important to consider that this work examined the effects of a single microbiota metabolite. In the true physiological environment, immune cells are undoubtedly in contact with a multitude of various metabolites at any given time. Thus, parameters such as developmental stage of the immune cell, metabolite concentration, and duration of exposure are likely to play a role in metabolite conditioning of immune cells.

Determining the cellular receptors to which particular metabolites bind will be useful in further understanding host-microbiota crosstalk. We have recently demonstrated that metabolite structure is extremely important in these interactions: indole and 5-hydroxy-indole, which differ by a single hydroxyl group, exhibit reciprocal effects on Treg and Th17 differentiation (Steinmeyer et al, in preparation). As such, it is likely that diverse metabolite classes exist that impose differential effects on immune cells in the gut. Future studies will be useful to investigate the effects of multiple, physiologically relevant metabolites in concert in order to better mimic the *in vivo* environment.

Closing remarks

Antigen-presenting cells in the gut provide a crucial function for the host, driving peripheral tolerance towards innocuous antigens and preventing aberrant inflammation. While host-derived factors are known to drive these properties in mucosal APCs, few specific microbiota signals have been identified that modulate mucosal APC characteristics. Previous work from our lab and others has demonstrated a protective role of the endogenous microbiota metabolite, indole, in the GI tract. The experiments described in this thesis uncover a previously unappreciated role for indole in instructing dendritic cells towards a mucosal phenotype and function, thus providing evidence for a single metabolite promoting peripheral tolerance (see Figure 59 for schematic representation). This revelation paves the way for future work in manipulating the microbiota for therapeutic potential in autoimmune and inflammatory disorders of the GI tract.

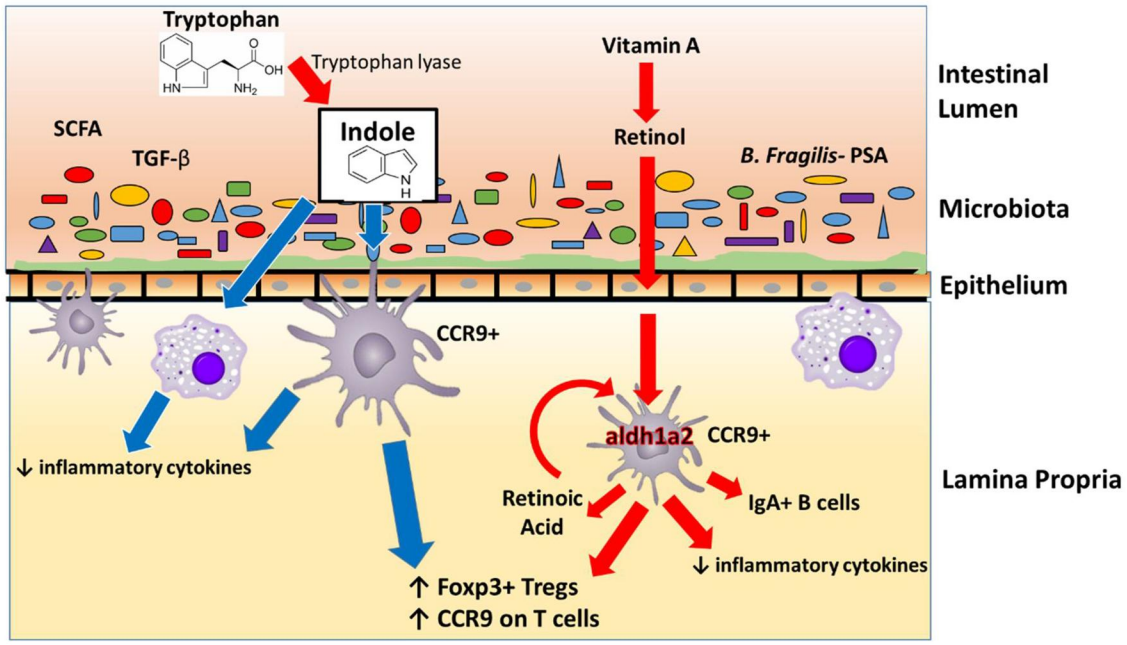

Figure 59. Indole is a microbiota signal that confers mucosal properties upon intestinal APCs.

Known gut-centric molecules which induce tolerogenic APCs include microbiota-derived short-chain fatty acids (SCFA) and *Bacteroides fragilis* with its outer membrane component, polysaccharide A (*B. fragilis*- PSA). The established, predominant host-derived factors which drive tolerogenic DC properties are the cytokine transforming growth factor-β (TGF-β) and the dietary vitamin A metabolite, retinoic acid (RA). Mucosal DCs express high levels of the enzyme aldehyde dehydrogenase 1a2 (aldh1a2) which catalyzes the RA-producing reaction, thus producing high concentrations of RA in the microenvironment. This RA acts directly upon DCs and CD4+ T cells to affect gene transcription for downstream effects including preferential induction of Foxp3+ Tregs, expression of gut-homing CCR9 receptors, class switching to IgA+ in B cells, and suppressed inflammatory responses to TLR stimuli. The tryptophan metabolite, indole, is an additional intestinal signal which induces mucosal properties in DCs in a manner independent of RA metabolism. Indole also inhibits pro-inflammatory responses to TLR stimuli in macrophages, promoting a tolerogenic environment.

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