METABOLITE SIGNALS FROM THE MICROBIOTA: INSTRUCTING T CELL FATE AND FUNCTION

A Dissertation

by

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ABSTRACT

The microbiota has a major impact on host health, and research suggests that the enzymatic capacity of the microbiota is important to mediate many of its effects on the host. Short-chain fatty acids (SCFAs) have recently been demonstrated to promote regulatory and effector T cell differentiation in the GI tract, but germ-free mouse studies suggest that additional signals from the microbiota are necessary in addition to SCFAs in order to recapitulate T cell lineage prevalence seen in colonized mice. We recently determined that a number of tryptophan (Trp) metabolites are present in colonized mice at high concentrations similar to SCFAs but not in germ-free mice. Therefore, we tested the ability of Trp metabolites to regulate T cell differentiation and investigated the interaction between Trp metabolites and SCFAs.

We found that the Trp metabolites indole and 5-hydroxyindole have anti- and pro- inflammatory effects on T cell differentiation, respectively, and some of these effects are dependent on the aryl hydrocarbon receptor. Additionally, a combination of indole, 5-hydroxyindole, and SCFAs produced unexpected outcomes during T cell differentiation, specifically promoting tolerogenic T cell differentiation without inhibition or augmentation of inflammatory T cell differentiation.

The powerful effects we observed *in vivo* on T cells conditioned with Trp metabolites *in vitro* suggests that these metabolites could be a useful conditioning agent for cellular therapeutics. In addition, the level of Trp metabolites and SCFAs being produced by the microbiota could be a useful marker for clinical manipulation of the microbiota during pathology such as Inflammatory Bowel Disease. To support the clinical implications of this work, we verified our major results in

human T cells.

Additionally, we established a protocol for generating a predictive model of microbiota metabolite interaction during T cell differentiation. We believe this tool will become increasingly useful as additional active microbiota-derived metabolites are identified and empirical determination of interaction between all types of active metabolites becomes increasingly cumbersome.

DEDICATION

To my mother, who taught me how to love unconditionally and stand tall in the face of adversity, and to my father, who taught me how to derive pleasure from an honest day's work.

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NOMENCLATURE

Trp	tryptophan
SCFAs	short-chain fatty acids
AhR	aryl hydrocarbon receptor
HDAC	histone deacetylase
GPR	G-protein coupled receptor
DMF	dimethylformamide
IND	indole
5HI	5-hydroxyindole
BUT	butyrate
CTRL	control
Th1	CD4+ T helper type 1
Th2	CD4+ T helper type 2
Th17	CD4+ T Helper Type 17
Treg	Regulatory CD4+ T Helper
NN	neural network
CFSE	carboxyfluorescein succinimidyl ester
PBSE	pacific blue succinimidyl ester

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW*

The GI tract mucosal immune system

Along with their close association with the microbiota, mucosal tissues—typified by the lungs, oral-nasal cavities, and the gastrointestinal (GI) tract—are functionally and anatomically specialized regions in the host that comprise the primary barrier to the external environment. It is not surprising then that the largest collection of immune cells in the body reside in the GI tract, and our group has published a recent review on mucosal immune cells in the GI tract and its relationship with microbiota-produced metabolites (1).

GI immune cells are organized as the gut-associated lymphoid tissue (GALT) that includes diffusely distributed immune cells in the lamina propria (LP); intestinal epithelial cell (IEC)-intercalating lymphocytes (IELs); secondary lymphoid tissue known as Peyer's Patches (PPs) and Colonic Patches (CPs); and solitary isolated lymphoid tissue (SILT) in the LP. A comprehensive description of the GALT architecture and function can be found in a recent excellent review (2).

Among the host lymphoid tissue, the GALT encounters the largest biomass of non-pathogenic microbes from both dietary intake and the endogenous microbiota. This unique antigenic challenge contributes to the phenomenon of oral tolerance, whereby nominal antigen acquired

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orally, in contrast to that encountered systemically, is effectively ignored, which illustrates the overall *modus operandi* promoting GALT homeostasis (3). Nevertheless, the GALT includes innate and adaptive immune mechanisms poised to respond to pathogenic infection. Consequently, the GALT—in conjunction with microbiota-dependent "colonization resistance" factors—clears most pathogenic microbes while avoiding aberrant inflammation (4, 5).

The GALT innate arm is composed of leukocytes such as macrophages, dendritic cells (DCs), and innate lymphoid cells (ILCs). In addition, other innate elements, including non-hematopoietic epithelial and stromal cells, produce protective cytokines and mucosal barrier components such as mucus and anti-microbial peptides (2, 6, 7). Macrophages and DCs possess critical antigen presenting cell (APC) and distinct immunoregulatory roles in the GI tract (8). Innate lymphoid cells (ILCs)—including natural killer (NK), lymphoid tissue-inducer (LTi), and other effector classes (9)—support pathogen clearance and tissue repair, and the current understanding of ILCs is well summarized in two recent articles (10, 11).

The GALT adaptive arm comprises B- and T-cells with particularly important roles for producing microbe neutralizing secretory IgA in the GI lumen and orchestrating the mucosal immune microclimate, respectively. Classical effector T-cells such as CD8+ cytotoxic T-lymphocytes (CTLs) and CD4+ T helper (Th) cells participate in mucosal immunity. Th1 (IFN-γ-producing, Tbet+), Th2 (IL-4-producing, GATA3+), Th9 (IL-9-producing, PU.1+, IRF4+), Th17 (IL-17-producing, RORγt+), Th22 (IL-22-producing), and regulatory (Treg) (TGF-β- and IL-10-producing, FOXP3+) T-cells reside and function in the GALT; however, Treg, Th17, and Th22 cells may have more prominent roles in the GALT. At mucosal surfaces, TGF-β is

abundant and specifically promotes differentiation of the peripheral Treg, Th17, Th22, and Th9 lineages (12-14), and these particular lineages are strongly influenced by additional signals through the aryl hydrocarbon receptor (AhR) that predominate in the GI tract (discussed below). T-cells receiving TGF-β signals plus IL-6 or TNF-α differentiate into Th17 or Th22 T-cells, respectively, and promote pathogen clearance and mucosal integrity (13). On the other hand, TGF-β plus IL-4 promotes Th9 differentiation whose function is similar to antibody-promoting Th2 T-cells (14). Further, it is important to note that both peripherally induced (TGF-β-dependent, pTregs) and thymically induced (TGF-β-independent, tTregs) Tregs are necessary for GI tract homeostasis (12), and even among pTregs, there is a division of labor, with phenotypically distinct subsets that respond specifically to dietary antigen and microbiota antigen (15). A more thorough discussion of general host factors involved in T-cell development and function can be found here (16). What is becoming increasingly evident is that non-host derived signals in the GI tract (i.e. from the microbiota) have profound regulatory influences on innate and adaptive cell functional fate.

T cells in the intestine are modulators of physiologic and pathologic inflammatory conditions

Physiologically, both pTreg and tTreg cells in the gastrointestinal tract promote long-term tolerance of commensal antigen derived from microbiota species (17, 18). This phenotype of Treg cells is important for maintaining the commensal population of non-host cells, and it is the result of a complex network of cytokine signaling mediated by intestinal epithelial cells in concert with innate and adaptive immune cells (19). In order to respond to pathogens, cells in the GALT are able to shift an immune response against a pathogen-derived antigen with precision

(20), highlighting the necessity of readily available pro-inflammatory as well as anti-inflammatory adaptive immune cells. The mechanisms behind the immune system's ability to identify commensal antigen are still being uncovered, but one prevalent hypothesis is that commensal microbiota species promote immune tolerance via the production of immunomodulatory metabolites.

Microbial insult coupled with genetic predisposition can disrupt the homeostatic induction of immune tolerance, leading to pathology such as inflammatory bowel disease (IBD) (21-24). The normally beneficial pathways of innate immune cytokine signaling and activation of adaptive immunity become pathologic (25, 26), contributing to increased pro-inflammatory cytokine production and stimulation of an inflammatory adaptive immune response against commensal antigen (27). Ultimately, this results in a chronic condition that is only transiently relieved by current therapeutics and can require surgical intervention (28). Current treatments have major side effects such as immune deficiency, and thus, a more selective immunosuppressive treatment, such as that offered by cell-based regulatory T cell therapy, could improve the quality of life for IBD patients (29, 30).

Adoptive transfer as an emerging therapeutic and its shortcomings

Cell-based therapy is a new frontier in clinical science with a number of promising features as well as hurdles to be cleared before successful implementation in a clinical setting. Unique features of this type of therapeutic compared to small-molecule and biological treatments currently available include the ability to perform complex biological tasks, potential for selectivity in their action, ability to deliver small-molecule and biological therapeutics in a

targeted manner and with a control circuit such that inter-patient genetic variability has a less pronounced effect on drug delivery than current treatments, and finally, cells offer a wide variety of malleable targets for bio-engineering. On the other hand, uncontrolled cell proliferation, migration and action in off-target tissue, incorrectly calibrated cellular action *in vivo* and a substantially increased complexity of the therapeutic all provide ample challenge for researchers to overcome before this treatment can be used to its full potential (29).

Adoptive transfer of Tregs as therapy for aberrant inflammation is a variation of cell-based therapy predicted to benefit a number of pathologies, including IBD, graft-versus host disease, type I diabetes, and a range of other auto-inflammatory disorders, based on studies in mouse models and preliminary clinical trials (30-37). The idea of adoptive transfer of Tregs in IBD treatment is based in part on clinical and experimental data demonstrating the balance of pro-inflammatory Th17 cells and anti-inflammatory Treg cells during physiologic GI immune surveillance and the shift in this balance towards pro-inflammatory Th17 cells during aberrant inflammation (38).

There are two major strategies currently being investigated for generating Treg cells for adoptive transfer: expansion of Tregs from the blood of a patient or induction of naïve T cells toward a Treg phenotype during activation *in vitro*. Currently, there is not a consensus on the best strategy for generating a Treg population for adoptive transfer, but due to the large importance of pTregs in colonic homeostasis, it can be argued that a naïve T cell population with a more diverse TCR repertoire than that of pre-existing Tregs in a patient might produce a more robust anti-inflammatory response in the GI tract once reintroduced into the patient. A related consideration

is the use of polyclonal vs. antigen-specific cell activation and expansion. Not only is the use of a polyclonal Treg population for transfer more feasible for widespread use, but the latest clinical trials suggest that polyclonal activation is a safe and potentially effective option (37). This dissertation will focus on polyclonal activation and induction of naïve T cells toward the regulatory phenotype, specifically denoted as *in vitro* induced Tregs (iTregs).

The current treatment options for IBD patients have drawbacks of non-specific immune suppression and/or toxic mechanisms (39). Developing a cell-based therapy has the potential to provide a more specific treatment option with fewer side effects for these patients. However, inconsistent induction of a homogeneously tolerogenic Treg population (35) and the loss of regulatory phenotype of Tregs after transfer (40) potentially limit clinical implementation.

Stability of Treg and Th17 phenotypes under inflammatory conditions in the GI tract

Both Th17 and non-thymic Treg cells are induced by TGF-β, and this is thought to serve as a basis for the reciprocal nature of their differentiation. Whereas the Th17 lineage will predominate in the presence of mostly pro-inflammatory cytokines (e.g. IL-1β, IL-6), cytokines such as IL-2 and metabolites specific to intestinal APCs (e.g. retinoic acid) will promote Treg development in the presence of TGF-β (41).

Investigators have described plasticity of T cell differentiation after activation of Treg and Th17 cells (42-44). Specifically, Tregs are able to switch to a pro-inflammatory, Th17 phenotype in the presence of inflammatory cytokines such as IL-1β, 6, and 23 in addition to the physiologically present anti-inflammatory cytokines of the GI tract (eg TGF-β) (45). This

observation has serious implications for iTreg adoptive transfer in the setting of IBD, because iTregs transferred to an inflamed gut could lose the regulatory phenotype and end up exacerbating the inflammation they were meant to resolve.

Metabolic signals have also been found to play a major role in separating Treg and Th17 differentiation (46). Specifically, Th17 cells are in a metabolically active state, relying more heavily than Tregs on glycolysis and glutamine breakdown for biosynthesis and maintenance of cellular function, whereas Tregs rely more heavily on fatty acid oxidation and are in a less metabolically active state (47). Indeed, inhibitors of mTOR, a potent metabolic activator, have promoted stability of adoptively transferred Tregs in inflammatory conditions *in vivo* (48).

The induction of Tregs in the GI tract and the functional importance of plasticity between Th17 and Treg lineage is not entirely clear. A better understanding of the role of microbiota influence in Treg induction and plasticity could provide a new paradigm by which investigators understand T cell biology in the GI tract, ultimately providing additional understanding necessary to fully develop iTreg adoptive transfer as a viable therapeutic in IBD treatment.

Microbiota composition in health and disease

The continuing advancement of metagenomic sequencing over the last 10 years has verified that the microbiota possess greater than 2 orders of magnitude more genomic content than humans, and microbiota dysbiosis (pertubations in abundance or diversity) is linked to pathology in a number of complex diseases, including IBD, obesity, diabetes, asthma, and psoriasis (49, 50).

Compounding potential exogenously-induced perturbations of the microbiota is the finding that microbiota variation between healthy individuals can be high, although it appears that on a population level microbiota composition can be broadly categorized into distinct enterotypes, which correlate with a person's geographical locale, education level and infant breastfeeding (51). Further, investigations of microbiome types possessing overall low or high gene content report that high gene content microbiomes have a more diverse repertoire of putative microbiota enzymatic function, and these high gene content hosts have a lower prevalence of complex disease (e.g., metabolic syndrome) (52). This observation supports the hypothesis that microbiota enzymatic richness and the end-product metabolites contribute important cues for optimal host immunophysiology.

Importantly, dietary components and nutrition strongly influence microbiota composition and disease. In perhaps the best direct evidence for this notion, humanized gnotobiotic mice fed a western diet (high fat, high sugar) have increased adiposity and altered microbiota composition compared to humanized gnotobiotic mice fed a standard diet (low fat, high plant polysaccharide), and transfer of the cecal microbiota from mice fed a western diet to germ-free mice maintained on a standard diet develop increased adiposity (53). However, a 12-week period of dietary intervention in 38 obese and 11 overweight individuals increased gene richness of the microbiota and decreased adiposity in low gene content individuals but failed to improve systemic inflammation markers to levels seen in the high gene content individuals, suggesting that microbiota composition also impacts the host response to dietary intervention (54).

Given its intimate relationship with diet, it is not surprising that the microbiota regulates host metabolome status both locally in the GI tract and systemically (55). On top of this, microbiota-specific enzymatic machinery produces unique metabolites—starting with substrates that originate in the diet—that modulate various pathology such as cardiovascular disease and cancer (56). It follows that increasing our understanding of microbiota metabolite diversity and function in the host has the potential to provide new targets for treating disease, but there are considerable obstacles in identifying the universe of microbiota metabolites. For example, it is conservatively estimated that the microbiota has at least 3 orders of magnitude greater enzymatic/biosynthetic potential than its human host (50); therefore, the challenges of microbiota metabolite testing are compounded by the sheer number of possible products for any given functional pathway.

To analyze the microbiota enzymatic repertoire, investigators initially focused on the abundance of different enzyme classes (57), however, enzymatic prevalence does not account for the community-level metabolic network that enzymes may belong to within the microbiota. Recent work by our team and others has validated the utility of placing metagenomic DNA gene reads into a computational model for metabolome network comparisons between samples and for hypothesis testing (55, 57, 58). For example, *Greenblum et al* used the Kyoto Encyclopedia of Genes and Genomes (KEGG) to categorize metagenomic DNA sequences from the microbiota of healthy, colitic, and obese individuals. The resulting enzymatic network revealed that microbiota gene abundance in obese and colitic individuals was primarily altered in the periphery of the enzymatic network (57). The periphery of the network of microbiota enzymatic machinery can be thought of as representing reactions that rely on substrates from the GI lumen or that produce metabolites that are not used by other microbiota enzymes; in other words, these enzymes are

likely to directly use or produce metabolites that are at the interface between host cells and microbiota. Thus, this observation suggests that complex diseases are linked with changes in microbiota metabolite signals received by host cells.

Whereas the above network approach correlates pathology to changes in the microbiota enzymatic network, our group's approach has focused on prediction of novel microbiota metabolite production *in silico* via network analysis of metagenomic DNA samples followed by targeted *in vivo* mass spectrometry-based metabolomics coupled to *in vitro* mechanistic analysis of host cell signaling pathways (58). Using this functional metabolomics workflow, we have identified novel aromatic amino acid metabolites, strictly microbiota produced, that signal through AhR (58). Of particular interest are two metabolites identified in this screen that will be investigated thoroughly in this dissertation: Indole and 5-HydroxyIndole.

Among the many potential metabolites selectively produced by the microbiota, the vast majority have not been tested or have unknown effects on homeostasis and physiology. Despite this, two major classes of metabolites have been found to have wide-ranging effects on host immunity and physiology: short-chain fatty acids (SCFAs) which have been long recognized as important energy substrates and regulators of cell function in the gut, and tryptophan (Trp) metabolites, which are rapidly emerging as one of the most bioactive classes of immunomodulators. The effect of these two classes of metabolites on T cell differentiation and function is the focus of this dissertation. See figure 1 for a diagrammatic representation of the current knowledge concerning the effect of these two metabolite classes on GI tract immunology.

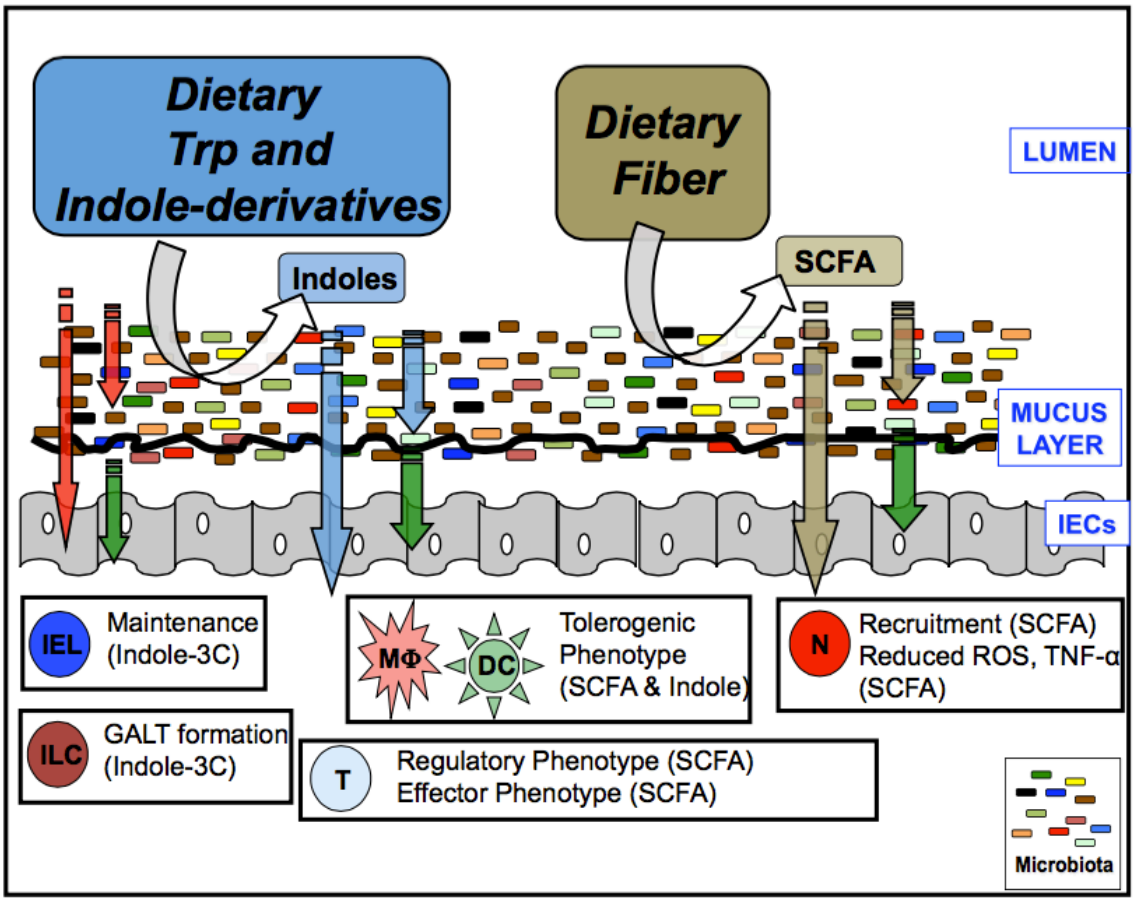

Figure 1. Tryptophan metabolites and short-chain fatty acids modulate GI tract immune cells.

Adapted from (1).

Short-chain fatty acids

Microbiota fermentation of dietary fiber, the non-starch indigestible poly- and oligo-saccharide portion of plants, is accomplished by microbiota encoded glucosidases to produce SCFAs that have long been recognized as an energy source for enterocytes, thereby maximizing the total energy yield from dietary intake. Butyrate, acetate, and propionate are the most prominent SCFAs in the gut, and each can range in concentration from 1 to 20 mM (59). However, only recently has molecular detail on the diverse regulatory effects of SCFAs, beyond simply augmenting nutritional yield, become apparent (60).

The SCFAs act as histone deacetylase (HDAC) inhibitors (which regulate DNA-histone coiling) as well as ligands for certain G-protein coupled receptors (GPRs): Olfr78, GPR41, GPR43 and GPR109a (61-66). The ubiquitous expression of HDAC enzymes in all nucleated cells, as well as the broad expression profile of these GPRs, underlies the varied physiological roles of the SCFAs, which include regulation of the nervous system (67-69), protection against colon cancer (70), and regulation of blood pressure and kidney function (66). However, a comprehensive understanding of the GPR- and HDAC-dependent functions of SCFAs is only beginning to be revealed.

With the exception of GPR109a, which can be activated by either the vitamin niacin or butyrate, SCFAs appear to be necessary for the homeostatic properties of the GPRs mentioned above. Indeed, acetate supplementation in the drinking water alleviated dextran sodium sulfate (DSS) colitis in WT but not GPR43 knockout mice (71, 72). In contrast, butyrate supplementation in the drinking water had no beneficial effect on DSS colitis (73). Additionally, feeding mice a high

fiber diet increased serum SCFA levels and alleviated house dust mite extract-induced allergic airway inflammation, while propionate supplemented drinking water alleviated allergic airway inflammation in WT but not GPR41 knockout mice (74).

Using GPR43 knockout mice, *Maslowski et al* (72) found increased disease pathology in multiple disease models including DSS colitis, K/BxN serum-induced inflammatory arthritis and OVA-induced allergic airway inflammation, and they proposed that increased inflammation in these disease models was mediated by neutrophils that lacked GPR43 signaling. GPR109a deficient mice had increased severity of DSS induced colitis, and loss of GPR109a resulted in decreased regulatory T cells in the colon as well as decreased IL-18 production in IECs (75). Indeed, DSS colitis was more severe in chimeric mice that lack GPR109a in either the hematopoietic or stromal compartment and most severe in total knockout mice (75). In contrast, *Kim et al* found that loss of either GPR41 or GPR43 in the non-hematopoietic stromal compartment resulted in decreased inflammatory response and delayed resolution of *C. rodentium* infection (76). These results suggest that pro- or anti-inflammatory effects of GPR activation, putatively via SCFAs, depend on the context and/or the cell type being activated.

IECs are likely the primary cell type exposed to SCFAs, and recent work has revealed that the barrier function of IECs is directly modulated by SCFAs. For example, butyrate increased transcript levels of MUC3 and MUC5B in the presence of glucose and increased transcript levels of MUC2, MUC3, MUC5AC, and MUC5B in the absence of glucose in IECs (77). Further, a synthetic HDAC inhibitor, trichostatin A (TSA), also increased transcript levels of MUC3 but no other MUC genes, suggesting HDAC inhibition may partially mediate mucus production induced

by butyrate (77). In addition, butyrate and propionate increased trans-epithelial electrical resistance in IECs, and this effect was recapitulated with TSA treatment (78). In addition to IECs at the mucosal barrier, HDAC inhibition by SCFAs likely mediates effects on innate hematopoietic cells. For example, propionate and butyrate decrease LPS-induced NF-κB activation and pro-inflammatory ROS and TNF-α production by neutrophils, likely by HDAC inhibition (79). Butyrate inhibits HDACs in bone marrow-derived macrophages and inhibits secretion of IL-6, IL-12 and nitric oxide, and this correlates with decreased IL-6, IL-12 and NO synthase transcript levels in LP macrophages isolated from the colons of antibiotic-treated mice given butyrate in drinking water (73).

In addition to SCFA modulation of innate immune cell types, recent work has demonstrated that SCFAs play a role in adaptive immune cell homeostasis. *Smith et al* demonstrated that acetate, propionate, and butyrate supplemented drinking water augments colonic Treg numbers, and this effect is mediated by GPR43-dependent accumulation of colonic Tregs in both germ free and specific pathogen free mice (80). In contrast, *Furusawa et al* (81) found that supplementing chow with butyrylated, but not acetylated or propionylated, starch induces colonic Tregs *de novo*. In agreement with these observations on the importance of the route of administration, *Arpaia et al* used FOXP3 CNS1 knockout mice, which are severely limited in peripheral Treg induction, to show that acetate and propionate in the drinking water primarily promote accumulation of colonic Tregs, and butyrate induces *de novo* generation of colonic Tregs only when applied by liquid enema or feeding butyrylated starch (82). Thus, the method of administration might alter SCFA bioavailability and the resultant physiologic effects. These studies highlight the variable signaling capacity of these three SCFAs, in that acetate and

propionate are most likely to activate GPR43 and promote colonic Treg migration/accumulation (80), whereas butyrate's strong HDAC inhibition is able to promote *de novo* colonic Treg generation (82).

In contrast to the homeostatic properties of SCFAs, acetate, propionate and butyrate may have properties that are pro-inflammatory. *Park et al* reported that SCFAs promote *in vitro* effector Th1 and Th17 differentiation (83). Interestingly, this study found that acetate supplemented drinking water increases colonic inflammatory Th1 and Th17 cells during infection with pathogenic *C. rodentium*, whereas in the absence of infection colonic IL-10+ T cells increase (83). However, it is not clear whether accumulation of existing T cells or *de novo* generation mediated the beneficial effects of acetate in this model. Further, this study found that *in vitro* T cells treated with acetate, propionate, or butyrate promoted phosphorylation of the ribosomal S6 protein, suggesting mTOR activation. Additionally, S6 kinase had increased acetylation after SCFA treatment, and this highlights the ability of HDAC inhibition to increase acetylation of histones as well as non-nuclear proteins (83). With growing appreciation of metabolic regulation of immunity—e.g., mTOR-mediated pathways (such as glycolysis) shape T cell differentiation lineage choice (84)—this newly revealed mechanism of SCFA signaling is intriguing and an important area for further study.

Tryptophan metabolites

The importance of microbiota products in shaping immune cell function in the GALT (e.g. SCFAs) suggests that alternate classes of microbiota metabolites may provide the host with necessary signals for proper development and homeostasis of the immune system. In fact,

emergent studies have established the importance of microbiota-derived Trp-metabolites for appropriate development and function of the immune system.

Beyond being a protein building block, the essential amino acid Trp is a substrate for host-dependent metabolic biotransformation into diverse chemoeffectors, e.g., the neurotransmitters serotonin and melatonin; indoleamine-2,3-dioxygenase(IDO)-dependent kynurenines (AhR ligands, (85)); and small amounts of the essential vitamin niacin. Beyond host-dependent metabolism of Trp, the microbiota perform unique catabolic biotransformations of Trp into several bioactive metabolites. Our team and others have determined that in the absence of a commensal microbiota many notable Trp metabolites are severely limited in both the GI lumen as well as serum, while levels of Trp in the serum are roughly doubled (55, 58). Furthermore, during clinical diseases associated with microbiota dysbiosis (e.g. IBD) Trp utilization in the GI tract is perturbed, causing increased luminal Trp levels and a concomitant decrease in Trp-metabolites (86, 87); this suggests that limiting concentrations of Trp-metabolites may trigger or exacerbate disease. Understanding the mechanistic immunophysiology of known and yet to be discovered Trp-metabolites will likely reveal new paradigms for microbiota-mediated communication with the host.

The properties of bioactive Trp metabolites appear to have special regulatory roles for signaling pathways in host immune cells. A recent study identified that microbiota-derived niacin as an agonist (as well as butyrate) of GPR109a promotes homeostatic IL-18 production in IECs and increases Treg prevalence in the colonic LP (75). However, an emerging paradigm reported by

our team and others is that a number of strictly microbiota-derived Trp-metabolites modulate AhR activity in immune cells (58, 88-90), which may play key roles in immunohomeostasis.

The AhR belongs to the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family of proteins, and although originally identified as a receptor for the industrial toxicant dioxin (91), its physiologic role is in adapting multicellular organisms to the environment (92). AhR is a ligand-inducible transcription factor that mediates cellular responses to low-molecular-weight chemicals by activating transcription of genes with promoters containing AhR binding sites, known as xenobiotic response elements (XRE). For mammals, the importance of the AhR for homeostasis of multiple immune cell types and proper GALT structure in the GI tract has gained interest in the last several years, with the first clear effects of AhR immunoregulation demonstrated on the balance of anti-inflammatory Tregs and proinflammatory Th17 cells (93, 94); a recent comprehensive review is available here (95). Furthermore, recent research has found that AhR knockout mice have normal thymic output, but maintenance of the IEL compartment is diminished (96). Further, lymphocyte-specific AhR-KOs (RAG1-cre x AhR-flox mice), revealed that intrinsic lymphocyte AhR activity led to a deficiency in the IEL population that exacerbated DSS colitis similar to what was seen in AhR KO mice (96). In a separate study, AhR KO mice revealed a defect in isolated lymphoid follicle formation, and this phenotype was also present in RORγT-Cre x AhR-flox mice. T cells were not necessary for follicle formation, therefore the investigators proposed that ILC-intrinsic AhR signaling was necessary for normal GALT development. Further, a diet deficient in AhR ligands recapitulated the defect in GALT development as seen in AhR KO mice and resulted in decreased RORγT+ ILCs, suggesting that AhR ligands may regulate optimal GALT development (97). To understand how the AhR

impacts RORγt+ ILCs requires further study, because ILCs are a critical junction between innate and adaptive immunity and necessary for physiologic tolerance of the microbiota (98).

The AhR has a number of ligands that have been identified and, although xenobiotic and exogenous natural AhR ligands may be acquired through the diet, the microbiota is likely the evolutionary and physiological meaningful source of AhR ligands in lower and higher vertebrates (99). Within the microbiota, the most proximal enzymatic pathway for Trp metabolites comes from species expressing tryptophan-lyase (100, 101), which directly catabolizes Trp to produce indole, an abundant metabolite found in both human and mouse fecal samples at high concentrations (58, 102-105).

Although indole has been known as a "by-product" of Trp catabolism, it was largely ignored as a bioactive molecule for decades (106). However, our team previously identified important microbiological properties for indole in decreasing chemotaxis, motility and IEC adhesion in enterohemmorhagic *E. coli* (107). Subsequently, we revealed that indole promotes IEC barrier integrity and expression of anti-inflammatory IL-10, while inhibiting inflammatory TNFα-induced IL-8 and NF-κB signaling (102). These protective effects of indole during inflammation were confirmed by *Shimada et al* who found oral indole therapy during DSS colitis in germ-free mice alleviated GI pathology, weight loss, and mortality (103). This study is notable, because it supports our own observations (102) and establishes that indole can function as a singular signal to promote homeostasis in the GI tract.

Undoubtedly, the pool of microbiota metabolites in mammals is highly complex (55). To deconvolute this inherent complexity, we recently used a computational network and metabolomics workflow to identify functional roles for indole and a number of endogenous Trp-metabolites/AhR ligands (58). Indeed, *Venkatesh et al* observed a novel interaction between indole and indole-3-propionate, another microbiota-derived Trp metabolite, enhances intestinal barrier integrity and inhibits inflammatory signaling in IECs through the pregnane X receptor (PXR) (108). This study found that indole-3-propionate plus indole activates PXR signaling in a reporter cell line, and they observed germ free mice and gnotobiotic mice colonized with metabolically inactive microbiota, in contrast to active, have exacerbated indomethacin-induced enteropathy. However, the investigators did not address any unique effects from indole alone, so it seems most likely that multiple signaling mechanisms explain the protective effects of indole and indole-3-propionate in IECs. Together, these studies suggest that distinct Trp-metabolites have both singular and combinatorial effects on multiple aspects of IEC signaling and physiologic response to environmental insult.

Another microbiota-derived Trp-metabolite, indole-3-aldehyde (I3Ald), promotes ILC production of IL-22 to protect against pathogenic infection, and this effect is dependent on the AhR (89). *Zelante et al* found that when host utilization of Trp was limited (IDO knockout mice), mice have enhanced resistance to *C. albicans* infection in the stomach, and this correlates with increased lactobacilli-mediated I3Ald production and ILC IL-22 production. Either increased dietary Trp or supplementation with I3Ald had a similar effect of reducing *C. albicans* infection load, whereas the protective effect of I3Ald supplementation was lost in AhR knockout mice (89).

In total, there is now compelling evidence that dietary Trp is a critical substrate for microbiota-dependent production of metabolites that regulate GALT development and promote homeostasis of many GALT-resident cells, usually via AhR signaling. However, due to the impressive variety and amount of bioactive Trp-metabolites produced by the microbiota (58, 88), it is clear that further investigation is necessary for a more comprehensive understanding of the role of Trp-metabolites in shaping host immune-homeostasis, particularly with respect to their properties as a consortium of metabolite signals, rather than isolated factors.

Computational modeling of T cell differentiation and function

The diverse functional characteristics of and plasticity between lineages of helper T cells poses a hurdle for developing Treg-based cellular therapy. Furthermore, the variety of microbiota metabolites with unique abilities to condition host immune cells means that effective utilization of microbiota metabolites as conditioning factors for cell-based therapy will require making sense of a very large, complex data set. Luckily, mathematical modeling of biological systems offers a robust approach to uncover non-intuitive dynamics that shape biological outcomes (109, 110).

In the biological system of Treg induction and transfer, metabolite signaling, cytokine signaling, as well as inter- and intra-cellular signaling are complex processes that are not completely understood and most likely shape the outcome of cellular therapy preparation. By utilizing a mathematical model that incorporates non-linear relations between experimental conditions in order to predict Treg properties (yield or stability) without requiring explicit modeling of the

signaling dynamics at play, we can predict biological behavior that would otherwise require rote empirical testing of a very large, unrealistic number of experiments.

Neural network models are unique in that they are generated from a limited set of experimental data, and without any explicit modeling of cellular signaling at play, they can determine nonlinear, multivariate relationships between culture conditions and potentially predict the outcome of T cell activation in the presence of multiple host cytokines and microbiota metabolite conditioning factors. The use of a feed-forward neural network, which acts as a nonlinear "black box" model of experimental data (111, 112), would complement the study of Treg cellular immunology in the context of microbiota-specific metabolites, an experimental system with many unknown signaling pathways.

Furthermore, neural network models allow for numerical analysis of experimental systems that have nonlinear relationships between a number of conditions (112), and recent modeling work suggests that systems of *in vitro* Treg induction demonstrate exactly these types of nonlinear relationships (42). For these reasons, Treg induction and stability are prime candidates for analysis via a neural network approach. Investigators have successfully employed this type of modeling to optimize or better understand biological systems (113-116), and the potential utility of these models for determining optimal T cell culture conditions will be addressed in this dissertation.

Developing microbiota metabolites as a tool kit for cellular therapy and clinical practice

Overall, we have a limited understanding of variable microbiota compositions and the concomitant metabolome signatures in health and disease. A recent study found that feeding butyrylated starch to specific pathogen free, but not germ free, mice increased de novo colonic Treg induction (81). This suggests that SCFAs alone might not be sufficient to regulate the GALT, and it is reasonable to predict that the net balance or specific interactions between SCFAs, Trp metabolites, and other microbiota metabolites is required to fully promote microbiota benefits. Thus, we propose that the presence and interaction between SCFAs and Trp-metabolites is essential for microbiota communication with the host. We will investigate the interaction of metabolites during T cell differentiation and how that interaction can produce unexpected outcomes.

In addition, the microbiota metabolites are a unique pool of diversely active molecules that could be useful for conditioning of human cellular therapeutics. In order to establish the feasibility of this process, we will test our major results of microbiota metabolite regulation of T cell differentiation in a human T cell experimental system. Returning to the mouse model, we will also investigate the effect of microbiota metabolite conditioning on cell function after transfer *in vivo*.

Furthermore, while it is possible to determine optimal culture conditions for generating stable and functional Tregs for transplantation using experimentation, this is not a feasible approach as the number of potential combinations of experimental parameters that can be varied (e.g., pro- and anti-inflammatory cytokines, microbiota metabolites, concentrations) is large and would

result in an extremely cumbersome experimental design. Therefore, we investigate the use of an integrated experimental/computational approach for optimization of Treg induction and Th17 attenuation conditions during *in vitro* activation and conditioning of T cells. We will use neural networks for modeling because of their suitability for representing poorly characterized responses such as those underlying microbiota regulation of CD4+ T cell differentiation. We hypothesize that the induction conditions determined by this integrated experimental/computational approach will result in increased Treg and attenuated Th17 populations *in vitro*, and stable Tregs *in vivo* after transplantation.

CHAPTER II

REGULATION OF CD4+ T CELL DIFFERENTIATION BY MICROBIOTA-DERIVED METABOLITES IN VITRO

Overview

The literature suggests that the microbiota plays a role in shaping CD4+ T helper cell lineage prevalence, both locally in the GI tract as well as systemically. In addition, dysbiosis is linked to autoinflammatory disorders such as Inflammatory Bowel Disease (IBD), which is in part mediated by pro-inflammatory CD4+ T cells. This suggests that the presence of a microbiota is not enough to promote a healthy adaptive immune response, rather certain microbe constituents making up a microbiota are important. We studied whether metabolites produced from specific members of the microbiota could alter T cell differentiation, and found:

1. Tryptophan (Trp) metabolites and short-chain fatty acids (SCFAs) have varied effects on T cell differentiation and can promote both pro-inflammatory and anti-inflammatory lineage differentiation.

2. The Trp metabolite indole has a unique ability to promote gut-homing markers and TGF-β production in FOXP3+ iTreg cells, whereas the SCFA butyrate promotes IL-10 production in FOXP3+ iTreg cells.

3. The effects of the microbiota-derived metabolites are mediated through separate pathways: Trp metabolites have multiple signaling mechanisms including the aryl hydrocarbon receptor (AhR) and mTOR, and SCFAs affect T cells through histone deacetylase (HDAC) inhibition.

4. The interaction of Trp metabolites and SCFAs result in unexpected outcomes during T cell differentiation.

5. Human T cell differentiation is also modulated by microbiota-derived metabolites.

Rationale

The study of germ-free and gnotobiotic mice has demonstrated that presence of a microbiota alters the state of the host immune system. This effect is far reaching and affects both innate and adaptive immune responses. As such, our lab and others have hypothesized that metabolites produced from the microbiota act as a new class of cytokines that can direct CD4+ T helper cell lineage choice during activation. We have previously found a number of tryptophan metabolites present in the GI tract of specific pathogen-free mice but not germ-free mice, and other labs have demonstrated that the short chain fatty acids acetate, propionate and butyrate are also found in decreased abundance in germ-free mice.

Results

Microbiota-derived tryptophan metabolites regulate T cell differentiation

We initially interrogated modulation of Th17 and Treg differentiation by a panel of dietary and microbiota-derived Trp metabolites determined from our previous work (58): indole, indole-3-carbinol, indole-3-acetamide, indole-3-pyruvate, indole-3-acetate, and 5-hydroxyindole (fig. 2). The concentrations tested were based on mass spectrometry readings of concentrations in fecal samples (58). We found that the first five of these metabolites all inhibited Th17 differentiation and either promoted or had no effect on Treg differentiation. On the other hand, 5-hydroxyindole had the opposite effect, blocking Treg differentiation and promoting Th17 differentiation. We

chose to further investigate the effects of two Trp metabolites strictly from the microbiota: indole, which had the most robust effect on T cell differentiation of the Treg-promoting Trp metabolites and 5-hydroxyindole.

We broadened the investigation to include four pathways of T cell differentiation: Th1, Th2, Th17 and Treg. Indole did not have any significant effect on Th1 or Th2 differentiation, but we observed a dramatic augmentation of Treg differentiation and inhibition of Th17 differentiation (fig. 3). On the other hand, 5-hydroxyindole promoted both Th1 and Th17 differentiation, blocked Treg differentiation, and had no effect on Th2 differentiation (fig. 4).

Since our differentiation assay used CD4+ CD25- conventional T cells, we verified these results in a more refined CD44lo CD62Lhi naïve T cell population to rule out any metabolite effects on residual effector or memory T cell function (fig. 43a). Furthermore, we verified that these observations were not the result of non-specific toxicity to inflammatory cells by indole or regulatory cells by 5-hydroxyindole by measuring proliferation of differentiated T cells with carboxyfluorescein succinimidyl ester (CFSE) dilution (fig. 43b) and using pac-blue succinimidyl ester (PBSE) to label dead cells (fig. 44).

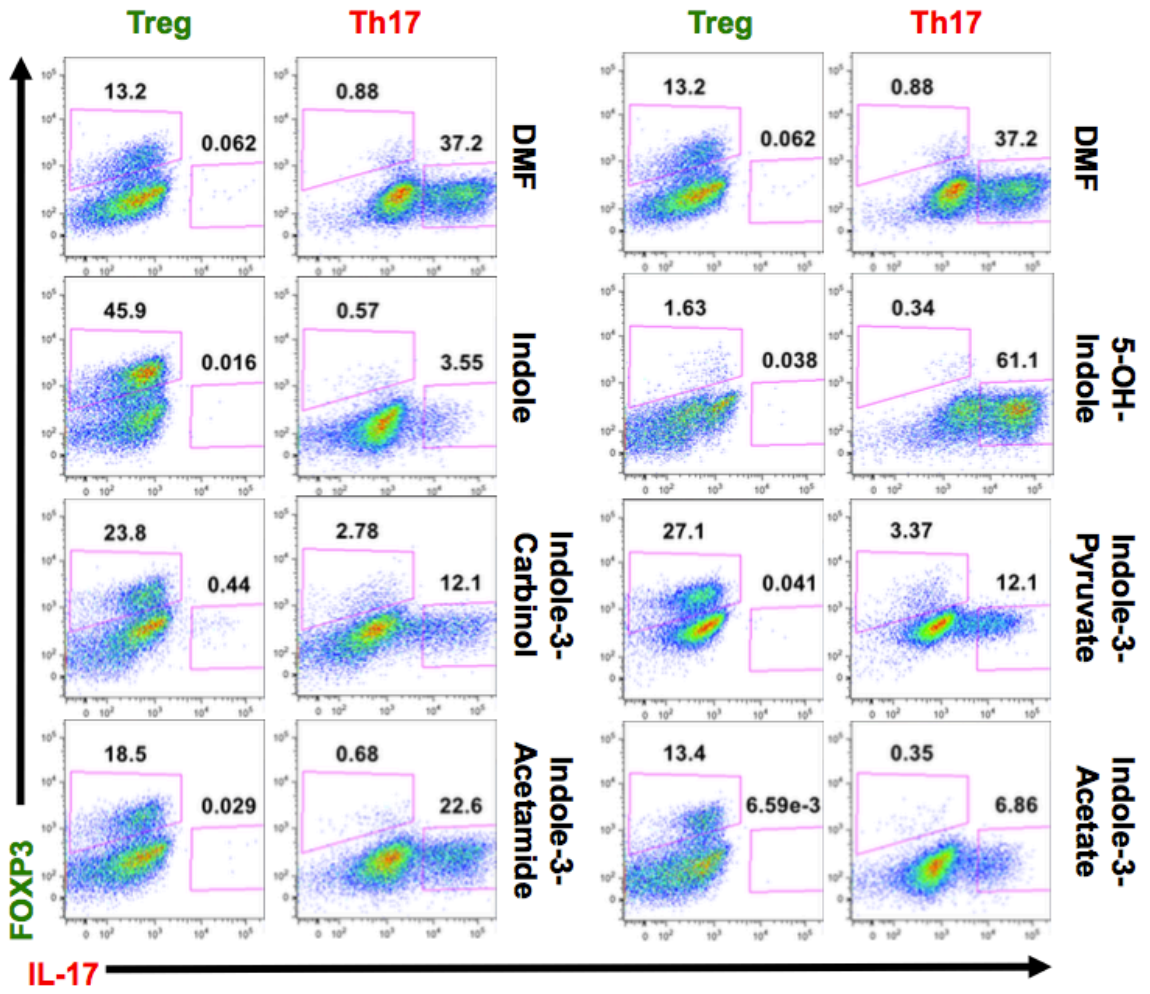

Figure 2. A panel of tryptophan metabolites have varied effects on Treg and Th17 differentiation *in vitro*.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Treg- or Th17- skew cytokines and indicated tryptophan metabolite or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining. Representative concentrations of metabolites are shown from a titration.

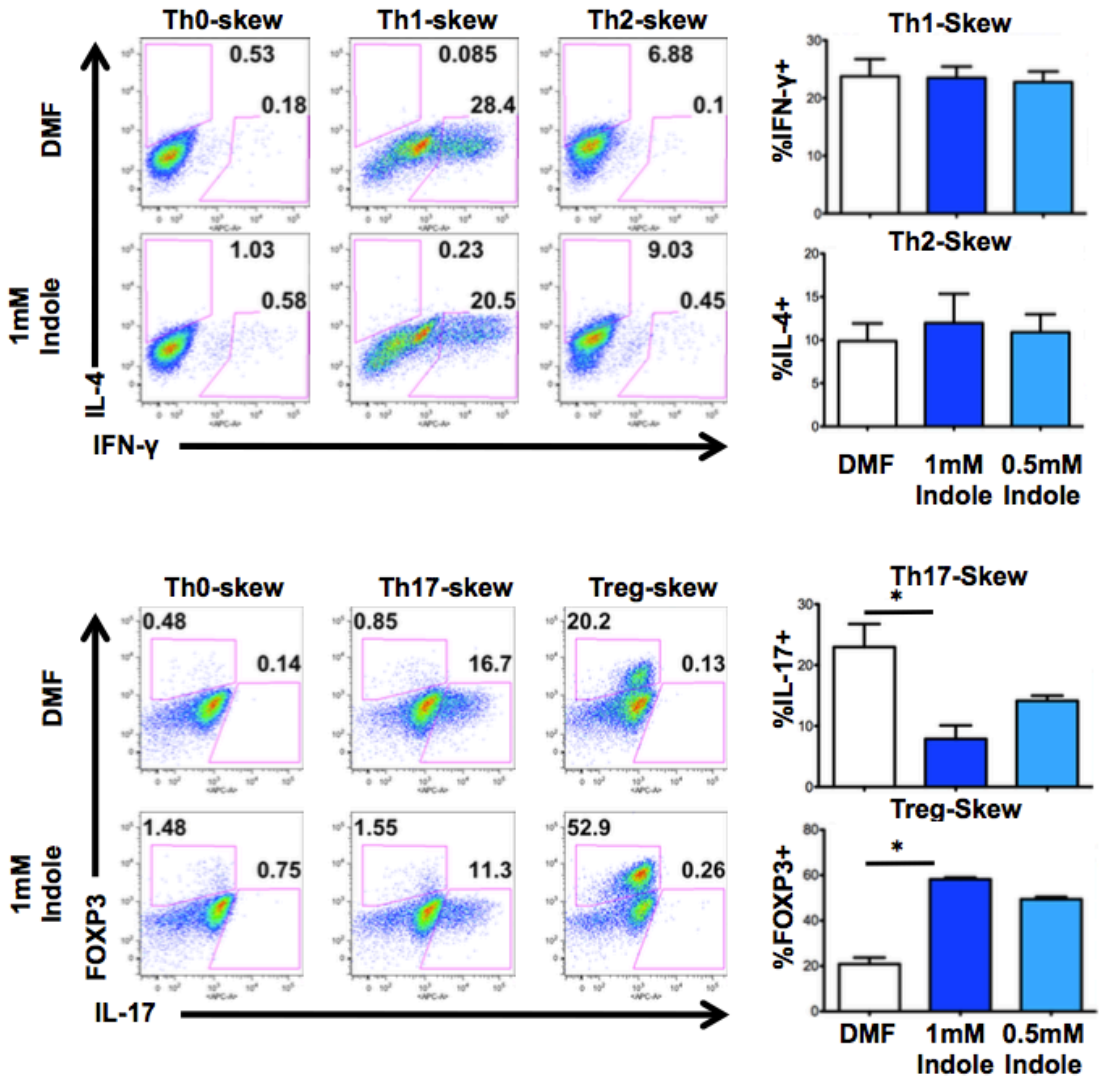

Figure 3. Indole has specific anti-inflammatory effects on Treg and Th17 differentiation *in vitro*.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th1-, Th2-, Treg-, or Th17- skew cytokines and indicated concentration of indole or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining. * denotes $p<0.05$ for one-way ANOVA followed by Dunnet's post-test.

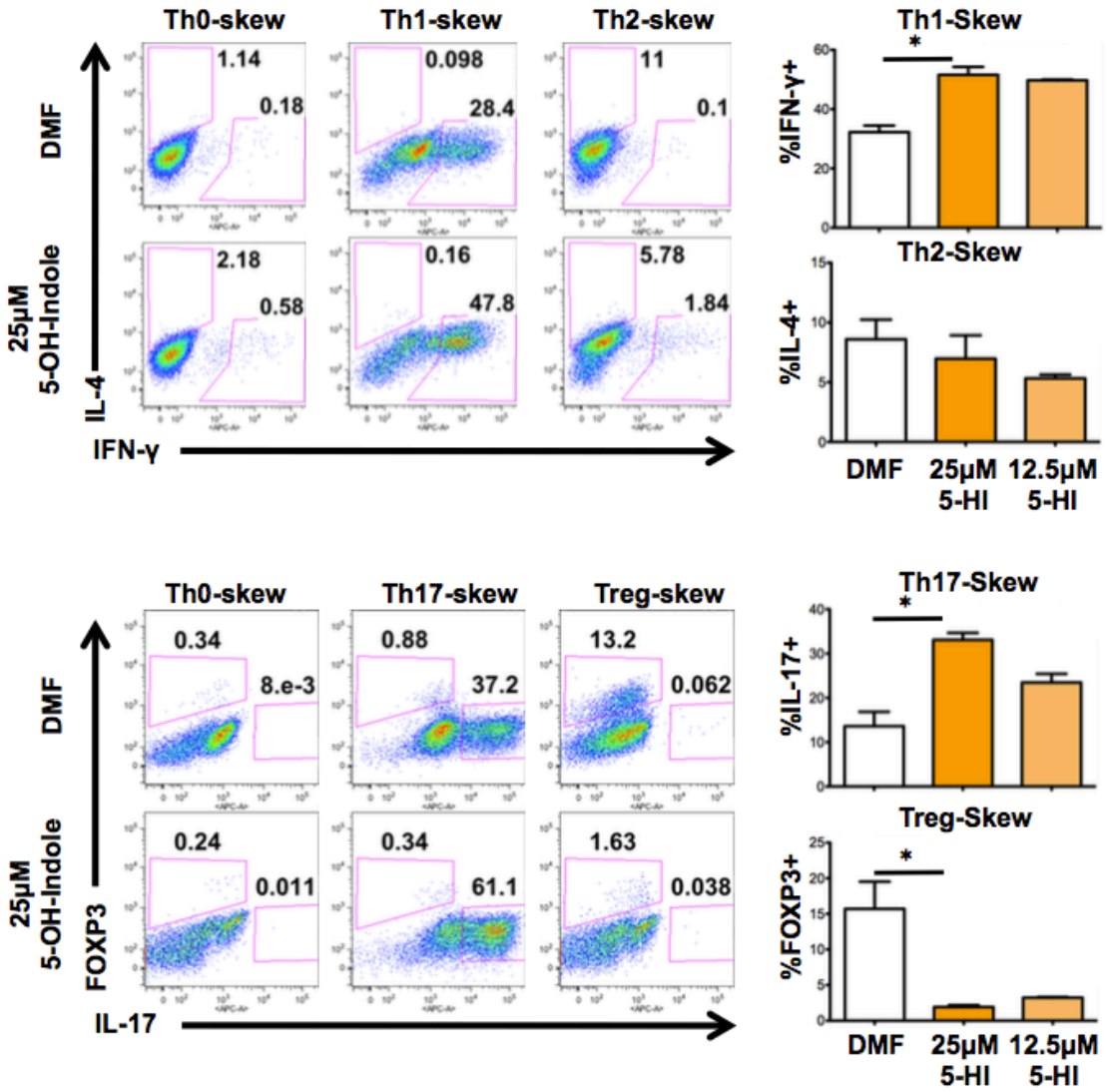

Figure 4. 5-Hydroxyindole has specific pro-inflammatory effects on Th1, Th17 and Treg differentiation *in vitro*.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th1-, Th2-, Treg-, or Th17- skew cytokines and indicated concentration of 5-hydroxyindole or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining. * denotes $p<0.05$ for one-way ANOVA followed by Dunnet's post-test.

Microbiota-derived short chain fatty acids regulate T cell differentiation

As has been reported previously, we observed augmented Treg differentiation with a low dose (0.1 mM) of butyrate and a higher dose (1 mM) of propionate, whereas even a very high dose (10-20 mM) of acetate did not augment Treg differentiation (fig. 5). Interestingly, these doses of SCFAs augmented Th1 and Th17 differentiation, with little effect on the Th2 lineage (fig. 5 and not shown), and this result has been replicated by another lab recently (83). Surprisingly, a high dose of butyrate (1 mM) promoted IFN-γ in every skew condition, and the expected outcome of Th2, Th17 or Treg differentiation was essentially blocked (fig. 6,7).

In the literature, groups have found similar results with 1 mM butyrate, specifically that it promotes IFN-γ and inhibits proliferation of T cells *in vitro* (117, 118); however, this aspect of T cell regulation by butyrate has been largely ignored in more recent studies (81, 82). Due to the unique ability of butyrate to promote Treg, Th1, and Th17 differentiation at a low dose similar to other SCFAs and also strictly promote Th1 differentiation at a higher dose, we focused on this SCFA for further study.

We verified the effects of butyrate on refined CD44lo CD62Lhi T cells (fig. 45). Additionally, we used PBSE live/dead staining and CFSE to verify that IFN-γ producing T cells treated with 1 mM butyrate were alive and proliferating during the differentiation assay (fig. 46, 47, 48, 49).

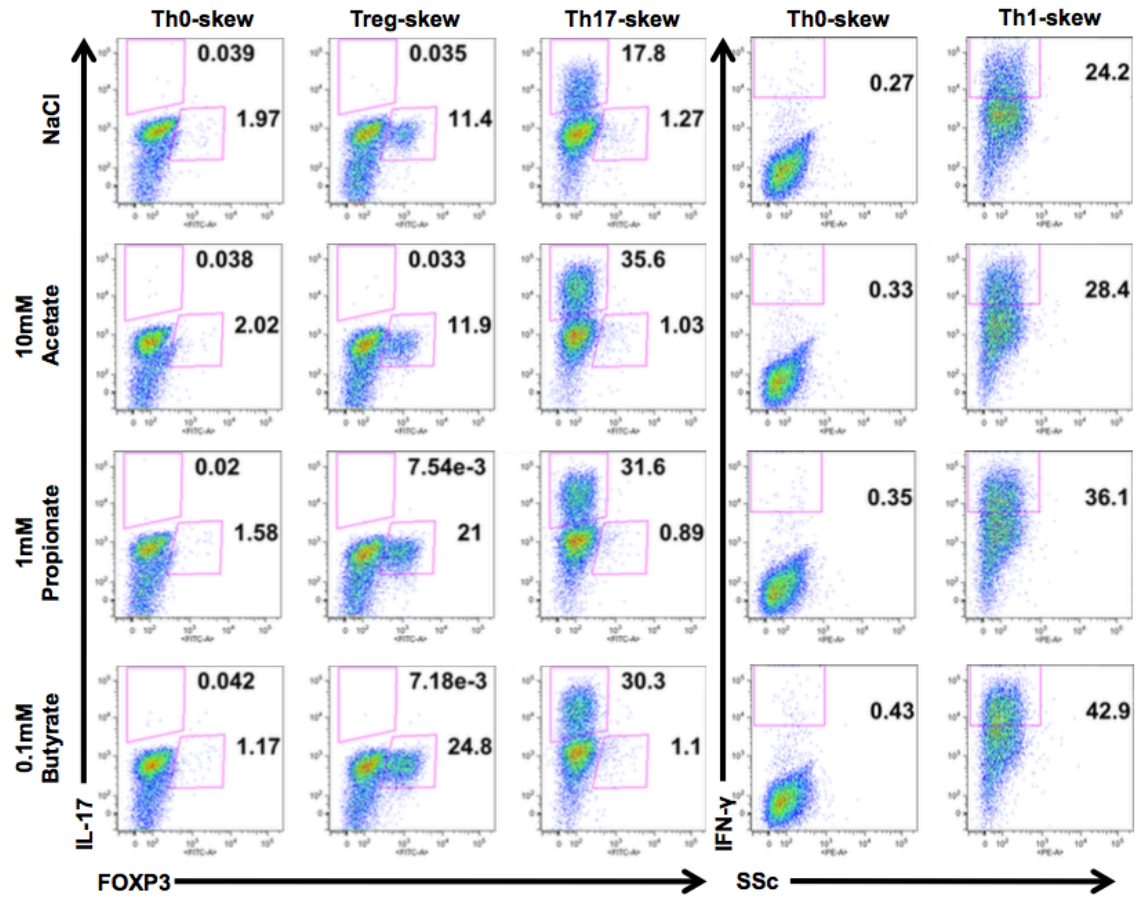

Figure 5. Short-chain fatty acids have varied effects on Th1, Th17 and Treg differentation *in vitro*.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th1-, Th17-, or Treg- skew cytokines and indicated concentration of SCFAs (acetate, propionate, or butyrate) or NaCl control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining.

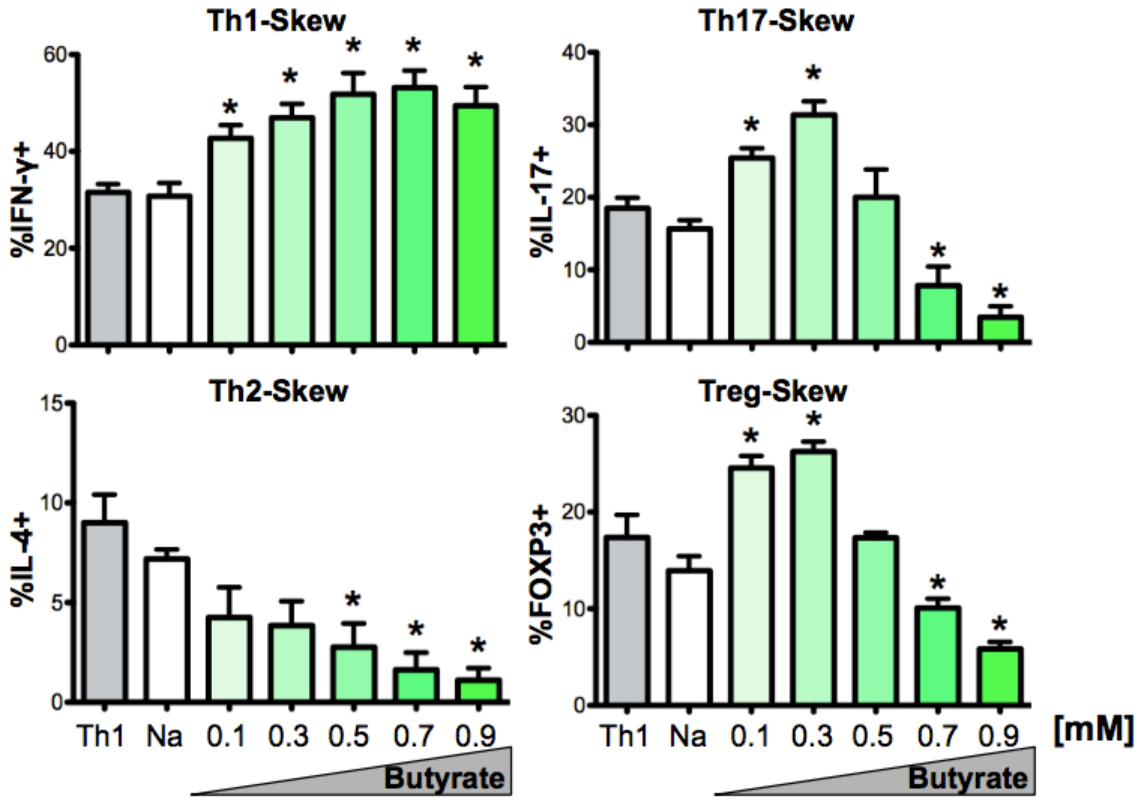

Figure 6. Butyrate promotes Th1 differentiation at all active, non-toxic concentrations, inhibits Th2 differentiation at high concentration, and promotes or inhibits Th17 and Treg differentiation depending on concentration.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th1-, Th2-, Th17-, or Treg- skew cytokines and indicated concentration of butyrate or NaCl control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining. * denotes $p<0.05$ for one-way ANOVA followed by Dunnet's post test compared to NaCl control.

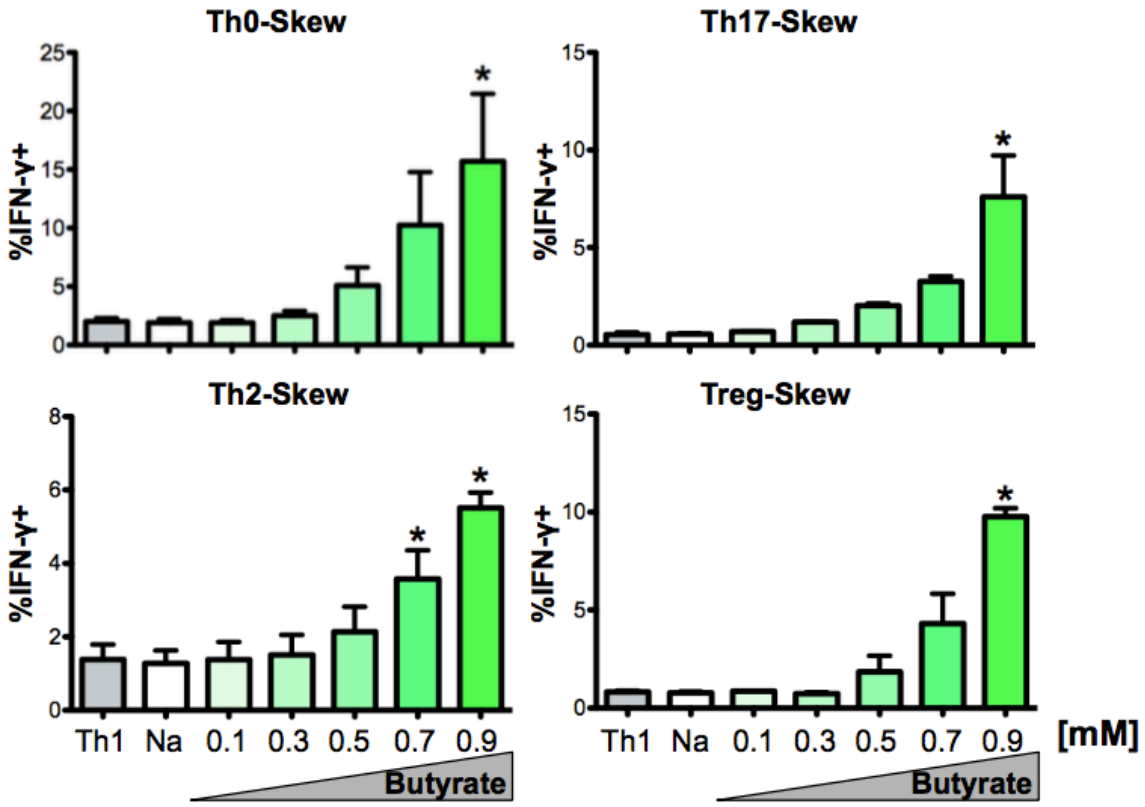

Figure 7. Butyrate promotes IFN-γ expression at high concentration independently of skew cytokine signaling.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th0-, Th2, Th17-, or Treg- skew cytokines and indicated concentration butyrate or NaCl control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining. * denotes $p<0.05$ for one-way ANOVA followed by Dunnet's post test compared to NaCl control.

Indole and butyrate uniquely alter the Treg phenotype

Previous studies have found that the host-derived vitamin A metabolite, retinoic acid (RA), not only promotes Treg differentiation, but also induces expression of gut-homing markers (α4β7 and CCR9) (119-121). This suggests that metabolites in the GI tract promote both generation and retention of Tregs. With this in mind, we wanted to further characterize the type of regulatory T cell promoted by microbiota-derived metabolites, so we performed surface staining for three homing markers that could promote retention in the GI tract of indole-conditioned Tregs, CCR9, CD103 and α4β7 (fig. 8). Furthermore, we performed surface staining for a number of classic Treg markers including CD152 (CTLA4), CD357 (GITR), and CD62L (fig. 9). We found that some of these markers were unchanged in indole-conditioned Treg cultures (CD152, CD357, CCR9 and CD103), but the gut-homing marker α4β7 as well as the lymph-node homing marker CD62L were significantly increased on FOXP3+ indole-conditioned Tregs. Interestingly, there was a synergy of α4β7 expression in Treg cultures treated with both indole and RA, suggesting that combined signals from microbiota- and host- derived metabolites could mediate the functional tolerance observed between the microbiota and host immune system. On the other hand, we found that these surface markers were not altered by butyrate (data not shown).

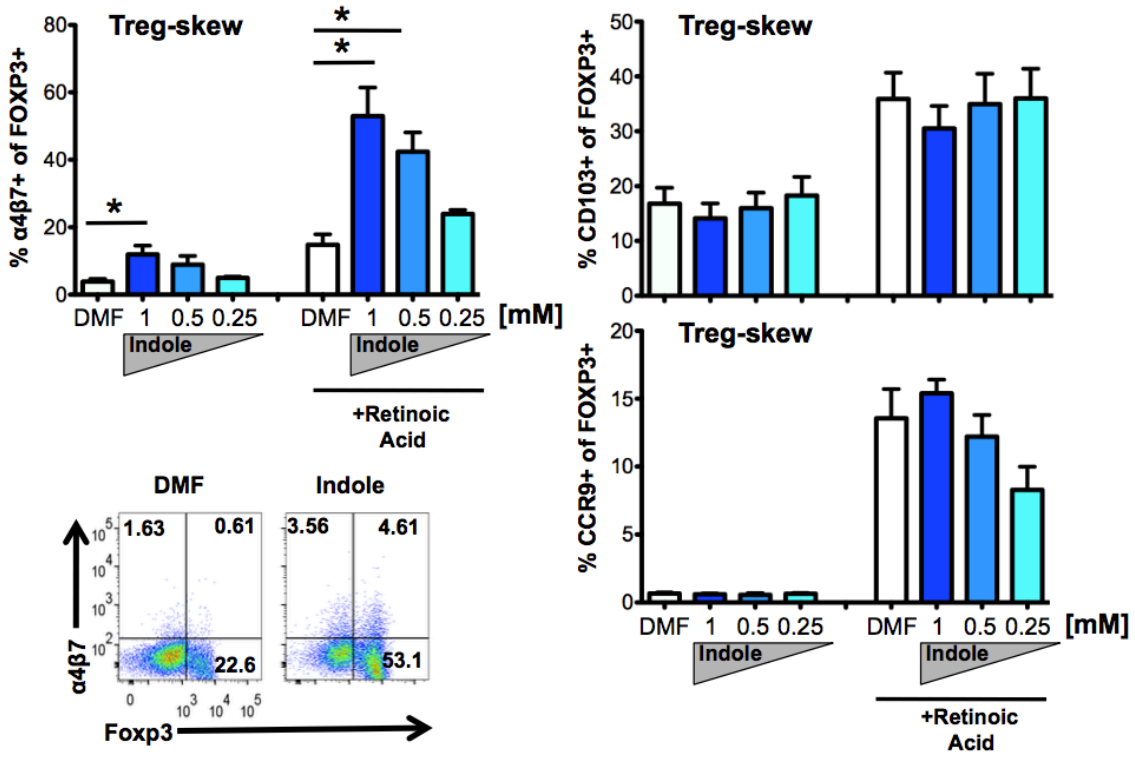

Figure 8. Indole promotes gut-homing marker α4β7 on iTregs independently and synergistically with retinoic acid.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Treg-skew cytokines and indicated concentration indole, 10 nM retinoic acid, or DMF solvent control. Data shown is percent positive for indicated gut-homing marker (α4β7 upper left, CD103 upper right, CCR9 lower right) of FOXP3+ iTregs. Representative data for α4β7 expression on iTregs is on lower left. * denotes $p<0.05$ for one-way ANOVA followed by Dunnet's post-test.

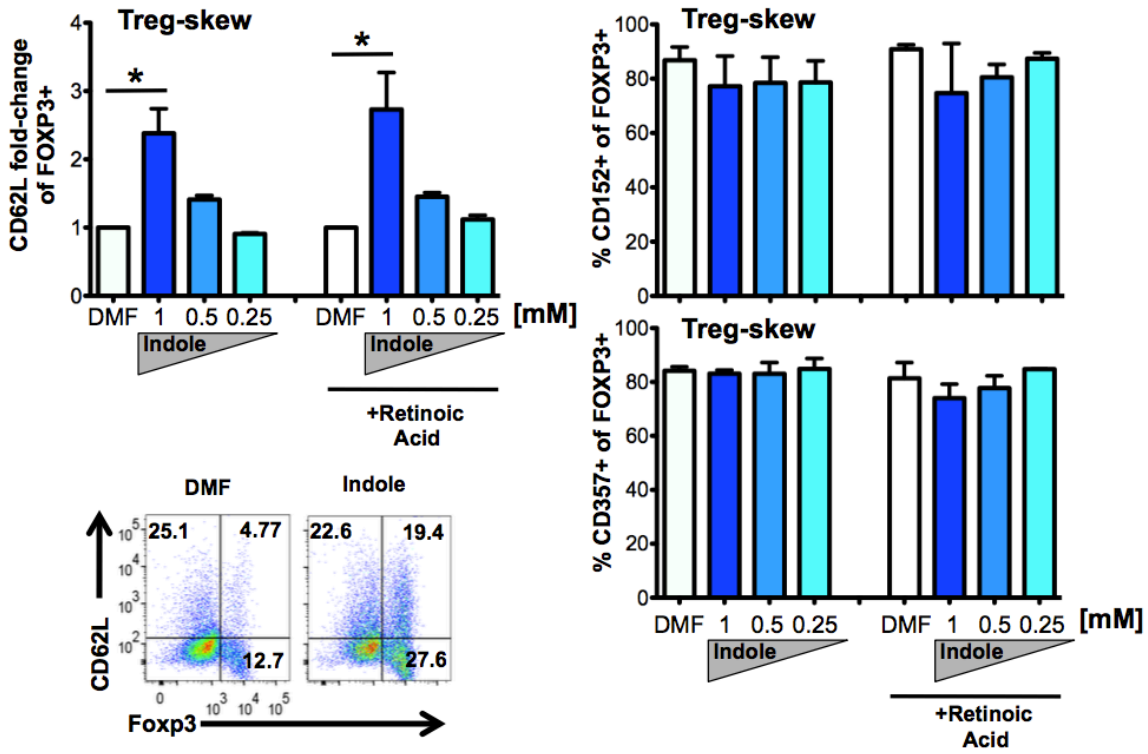

Figure 9. Indole promotes lymph-node homing marker CD62L on iTregs.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Treg-skew cytokines and indicated concentration indole, 10 nM retinoic acid, or DMF solvent control. Data shown is percent positive for indicated surface marker (CD62L upper left, CD152 upper right, CD357 lower right) of FOXP3+ iTregs. Representative data for CD62L expression on iTregs is on lower left. * denotes $p<0.05$ for one-way ANOVA followed by Dunnet's post-test.

Among the many mechanisms of Treg suppression, cytokine expression (particularly IL-10 and TGF-β) is thought to play a crucial role in the ability of Tregs to promote homeostasis in the GI tract (122, 123). In fact, different phenotypes of tolerogenic T cells have been described that express more IL-10 than TGF-β (FOXP3- Tr1 cells), and our data suggests that the microbiota not only promotes FOXP3+ Treg generation as other labs have seen but also alters the Treg phenotype. Therefore, we wondered if microbiota metabolite-conditioned Tregs had altered suppressive capacity and/or cytokine expression. Using a GFP-FOXP3 transgenic system, we purified FOXP3+ Tregs from cultures treated with indole, butyrate, or DMF solvent plus NaCl control; and these cells were cultured with freshly isolated CD8+ T cells in the presence of αCD3 cross-linking antibodies. In terms of IL-2 production, a marker for T cell activation, we found that indole- and butyrate-conditioned Tregs were no more or less suppressive than control iTregs or freshly isolated nTregs (fig. 10). On the other hand, we found that compared to control Tregs, indole-conditioned Tregs produced 2-fold more soluble TGF-β and produced no detectable IL-10 (fig. 10). In contrast, butyrate-conditioned Tregs had no change in TGF-β production but did produce more IL-10 (fig. 10). Additionally, we observed with intracellular FOXP3 staining intensity as well as GFP fluorescence intensity that indole-conditioned Tregs expressed more FOXP3 on a per-cell basis (fig. 10 and data not shown). Previous work has established a positive correlation between stability and FOXP3 expression level in a Treg cell (124), so this further supports the hypothesis that indole confers a unique phenotype on Tregs induced from naïve T cells.

Taken together, we see that indole not only promotes Treg differentiation but conditions Tregs towards a unique phenotype, able to home to the gut and draining lymph nodes and also

potentially promote Treg generation and tissue repair by increased TGF-β production. Furthermore, the data suggests that butyrate-conditioned Tregs have a unique function as well, producing increased amounts of IL-10, an important anti-inflammatory cytokine in the GI tract. Considering previous evidence showing that retinoic acid also mediates a unique Treg phenotype, it seems likely that the metabolite repertoire a T cell encounters during activation has important consequences for the immune status of an individual.

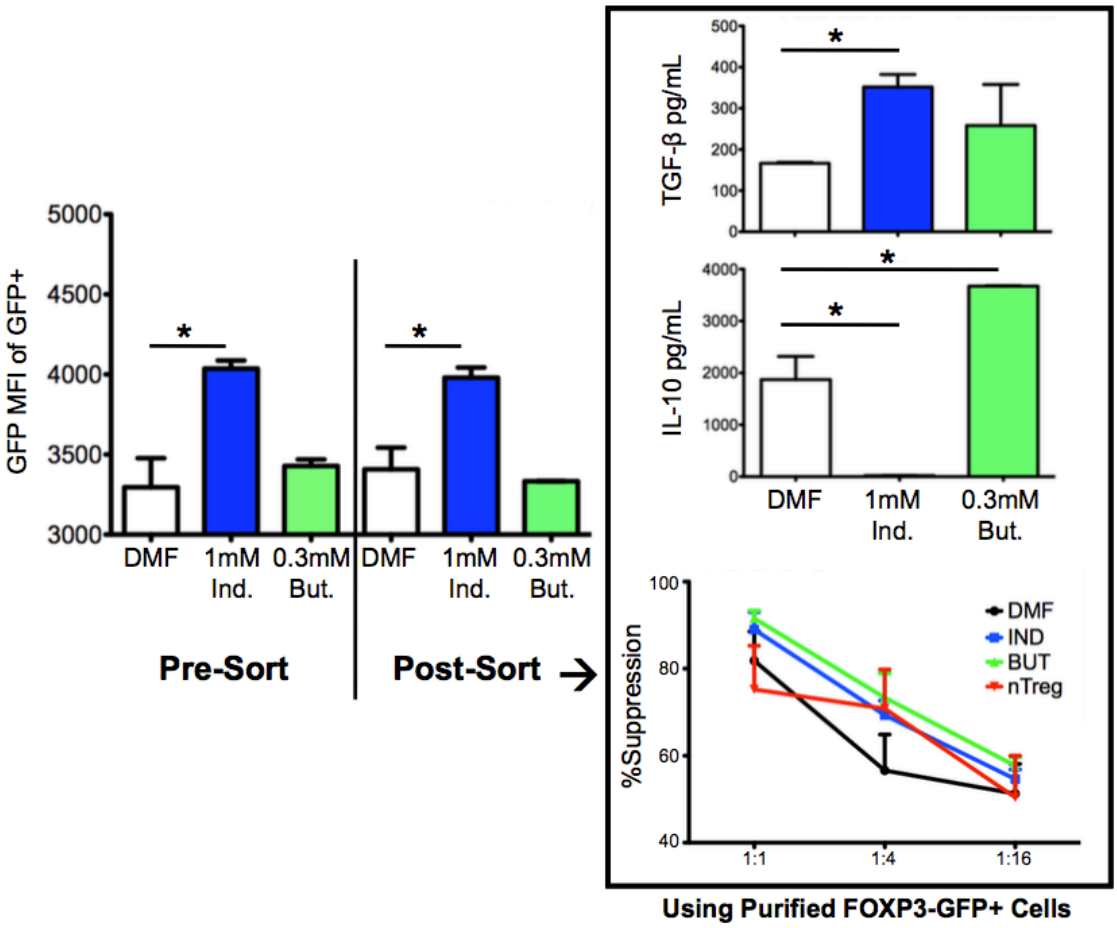

Figure 10. Indole and butyrate affect cytokine expression, but not suppression of CD8+ effector T cell production of IL-2, by purified FOXP3-GFP+ metabolite-conditioned iTregs.

CD4+ CD25- T cells from FOXP3-GFP transgenic mice sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Treg- skew cytokines and indicated concentration indole, butyrate, or DMF solvent control. At the end of 72 hours, FOXP3+ iTregs from culture were sorted for use in functional assays. * denotes $p<0.05$ for one-way ANOVA followed by Dunnet's comparison test to DMF solvent control.

Mechanism underlying the effect of tryptophan metabolites on T cells

To better understand the effect of indole and 5-hydroxyindole on Treg and Th17 differentiation, we interrogated TGF-β, IL-2 and IL-6 signaling pathways, all of which are important Treg- and/or Th17- skew cytokines. We began by performing a simple titration of these three cytokines with or without indole or 5-hydroxyindole (fig. 11). Interestingly, we found synergistic promotion of Treg differentiation with TGF-β and indole. Specifically, we observed a low dose of TGF-β that does not induce Tregs alone is able to robustly induce Tregs in the presence of indole. Additionally, we found that a low dose of IL-6 unable to induce Th17 cells alone does promote Th17 differentiation in the presence of 5-hydroxyindole.

In order to verify the role of indole and 5-hydroxyindole as modulators of downstream signaling from Treg- and Th17- skew cytokines, we performed phospho-staining experiments. Mesenteric lymph node cells were cultured overnight with indole or 5-hydroxyindole, followed by stimulation with cytokine and subsequent staining for downstream signaling protein phosphorylation. CD4+ T cells from the tissue culture treated with indole had increased expression of the TGF-β receptor and phosphorylation of SMAD2/3 after TGF-β stimulation, whereas 5-hydroxyindole had no significant effect on either readout (fig. 12). Neither indole nor 5-hydroxyindole affected STAT5 phosphorylation downstream of IL-2 stimulation (not shown). On the other hand, indole inhibited both IL-6R expression and phosphorylation of STAT3 in response to IL-6 (fig. 12). 5-hydroxyindole had no effect on IL-6R levels, but did show increased pSTAT3 (fig. 12). These results further support the hypothesis that indole and 5-hydroxyindole modulate the effects of host skew cytokines during T cell differentiation.

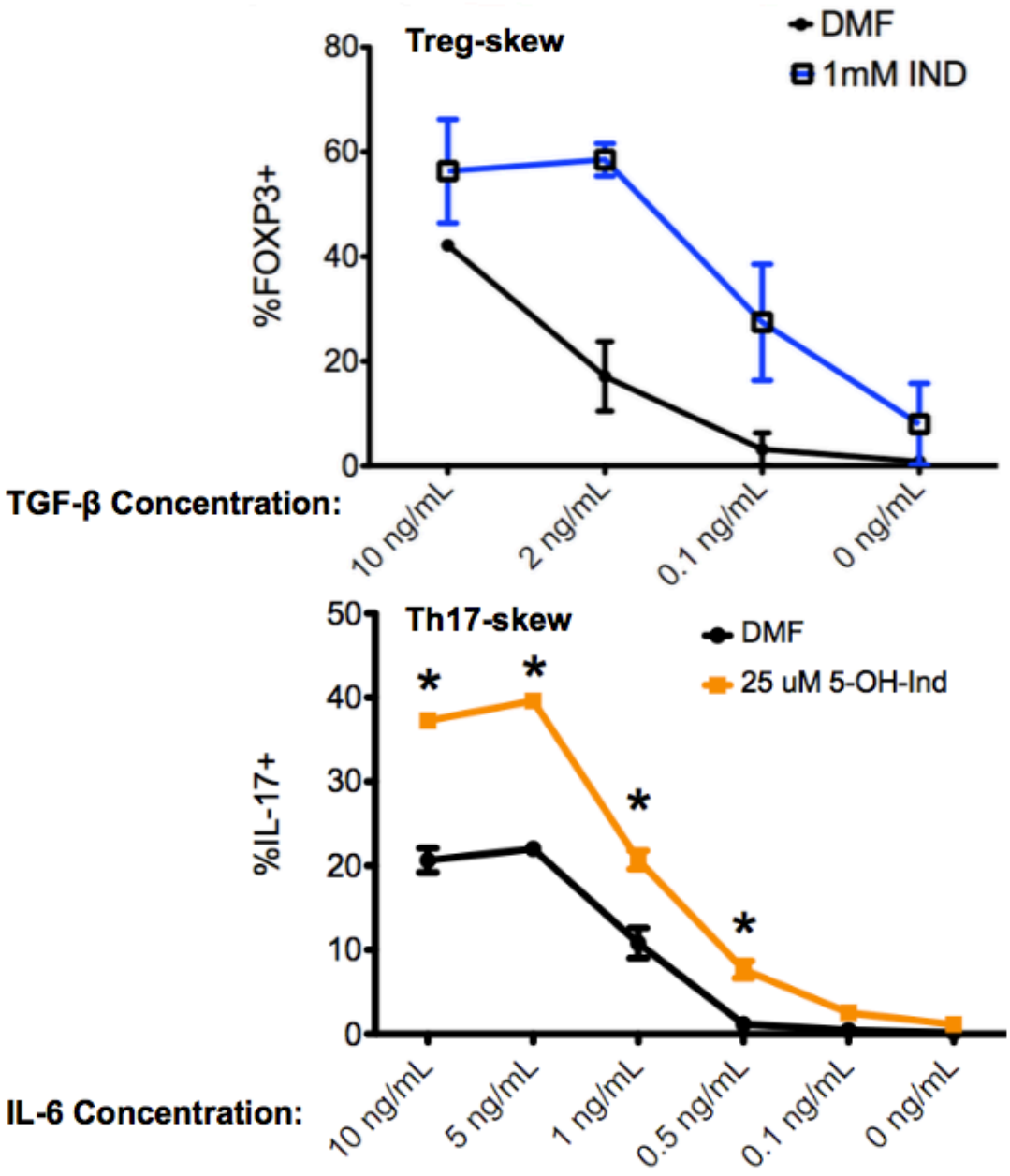

Figure 11. Indole and 5-hydroxyindole promote Treg and Th17 differentiation, respectively, at sub-optimal skew cytokine concentrations.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th17 or Treg- skew cytokines at indicated concentrations and indole, 5-hydroxyindole, or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining. * denotes $p<0.05$ for student's t test compared to DMF control.

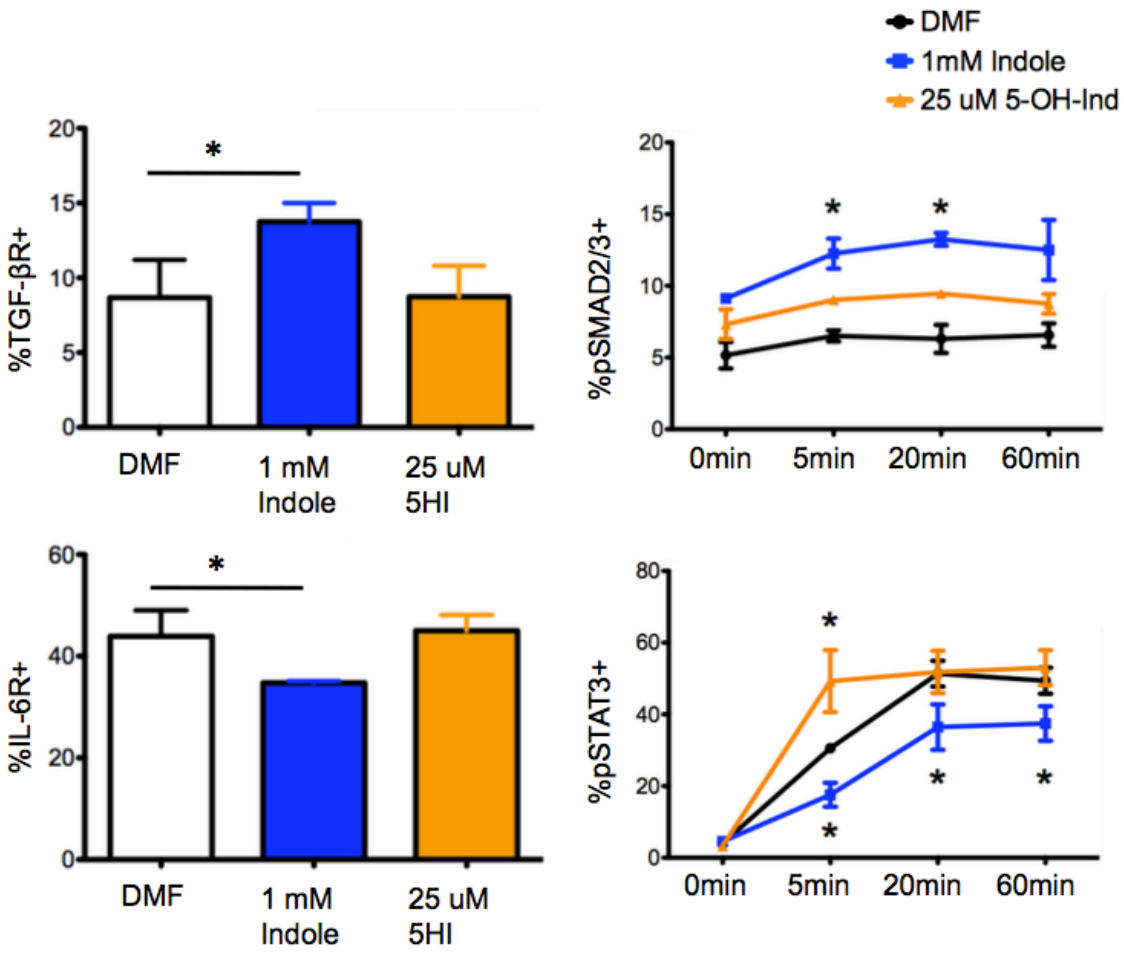

Figure 12. Indole and 5-hydroxyindole modulate signaling downstream of TGF-β and IL-6.

Whole splenocytes were incubated overnight with indole, 5-hydroxyindole, or DMF. Cells were stained for CD4, IL-6 receptor, and TGF-βR (left two panels). Additionally, TGF-β or IL-6 were added to cultures at indicated time points, followed by phospho-staining for pSTAT3 and pSMAD2/3 (right two panels). All percentages reported are of CD4+ splenocytes. * denotes $p<0.05$ for student's t test compared to DMF control.

We have previously shown that indole and a number of other microbiota-derived Trp metabolites modulate signaling through AhR (88), so we proceeded to test our observations in AhR knockout mice in order to identify a potential host receptor that mediates the effects of these Trp metabolites. Indeed, we found that wild-type, but not AhR knockout, T cells demonstrated augmented Treg and diminished Th17 differentiation in response to indole (fig. 13). However, we also found that both wild-type and AhR knockout FOXP3+ Tregs expressed increased levels of CD62L and α4β7 when cultured in the presence of indole (fig. 14). 5-Hydroxyindole only had a partial loss of Th17 promotion in AhR knockout T cells with fully intact promotion of Th1 and inhibition of Treg differentiation (fig. 15). Despite the apparent AhR-independent Treg inhibition by 5-hydroxyindole, Cyp1a1 transcript levels—a marker for AhR activity—significantly increased in 5-hydroxyindole treated Treg cultures of wild-type T cells, but AhR knockout T cells had no detectable Cyp1a1 transcript from any skew cytokine or metabolite treatment (fig. 16 and data not shown). These results suggest both Trp metabolites might modulate AhR signaling, but an additional AhR-independent signaling mechanism underlies some effects of both Trp metabolites during T cell differentiation.

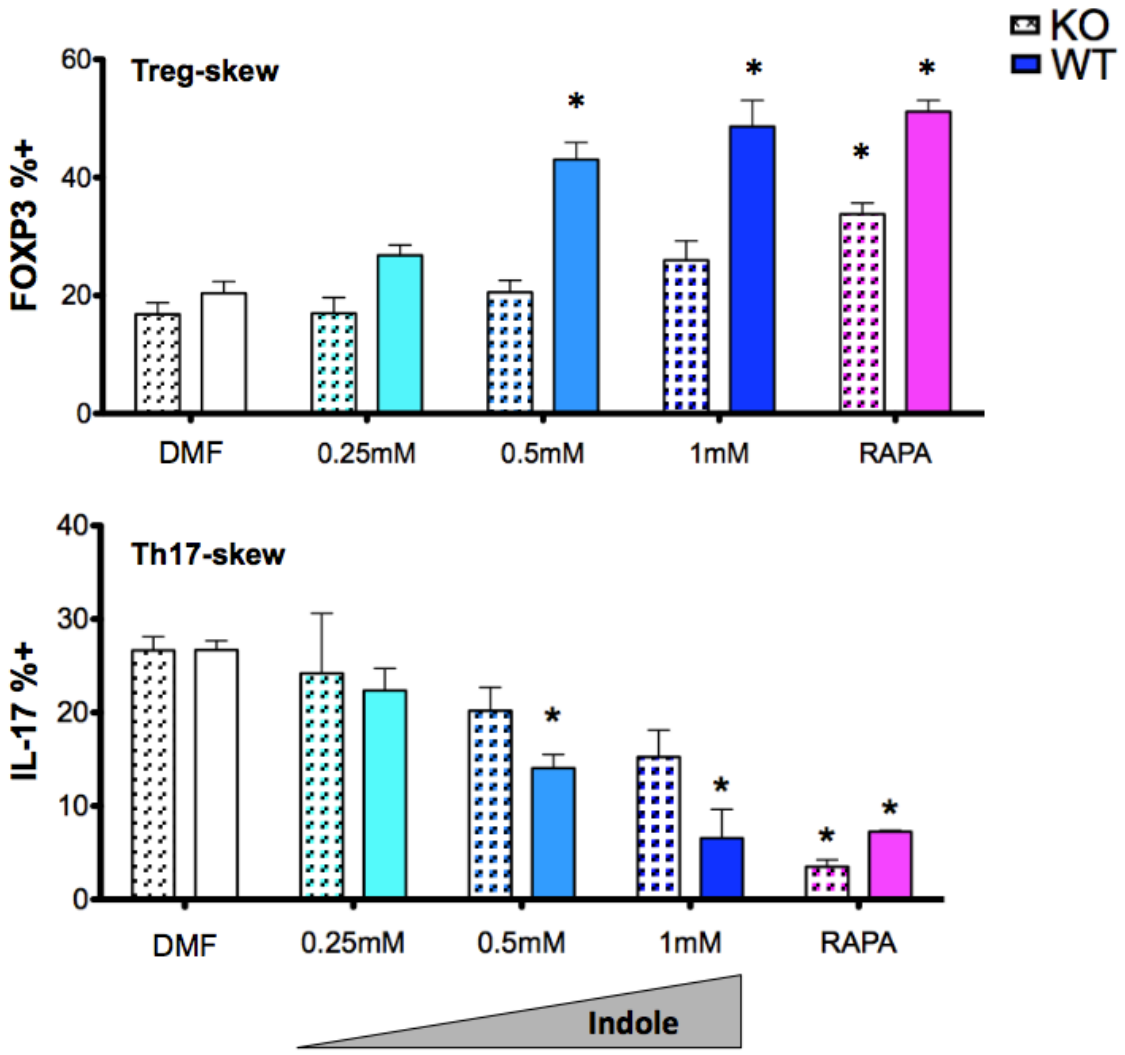

Figure 13. Indole regulation of CD4+ T cell differentiation is aryl hydrocarbon receptor dependent.

Wild-type or AhR knockout CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th17- or Treg- skew cytokines and rapamycin (RAPA), indole or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining. * denotes $p<0.05$ for one-way ANOVA followed by Dunnet's comparison test to DMF solvent control of respective WT or AhR KO.

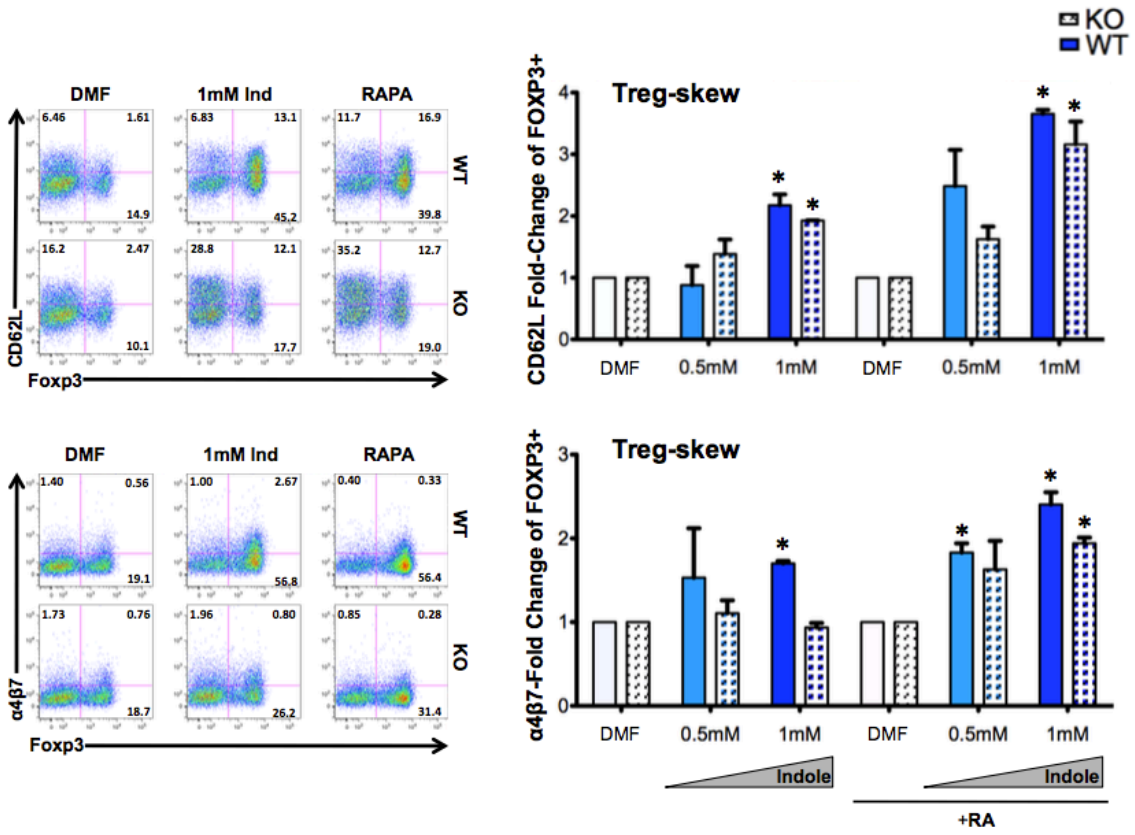

Figure 14. Indole promotes lymph node- and gut-homing markers independently of the aryl hydrocarbon receptor.

Wild-type or AhR knockout CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Treg- skew cytokines and indicated concentration indole, 10 nM retinoic acid, or DMF solvent control. Data shown is percent positive for indicated surface marker (CD62L upper panels, α4β7 lower panels) of FOXP3+ iTregs. * denotes $p<0.05$ for one-way ANOVA followed by Dunnet's comparison test to DMF solvent control of respective WT or AhR KO with or without retinoic acid.

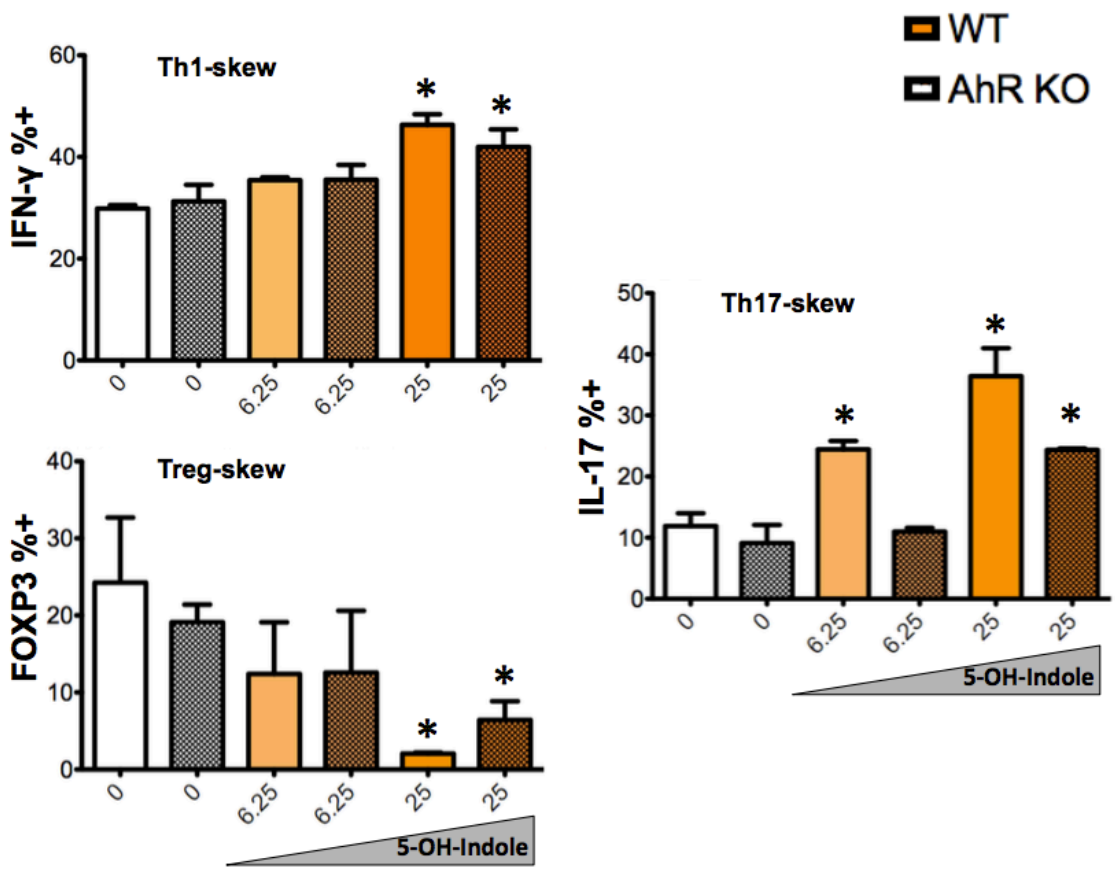

Figure 15. 5-Hydroxyindole regulates T cell differentiation partially independently of the aryl hydrocarbon receptor.

Wild-type or AhR knockout CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th1-, Th17- or Treg- skew cytokines and 5-hydroxyindole or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining. * denotes $p<0.05$ for one-way ANOVA followed by Dunnet's comparison test to DMF solvent control of respective WT or AhR KO.

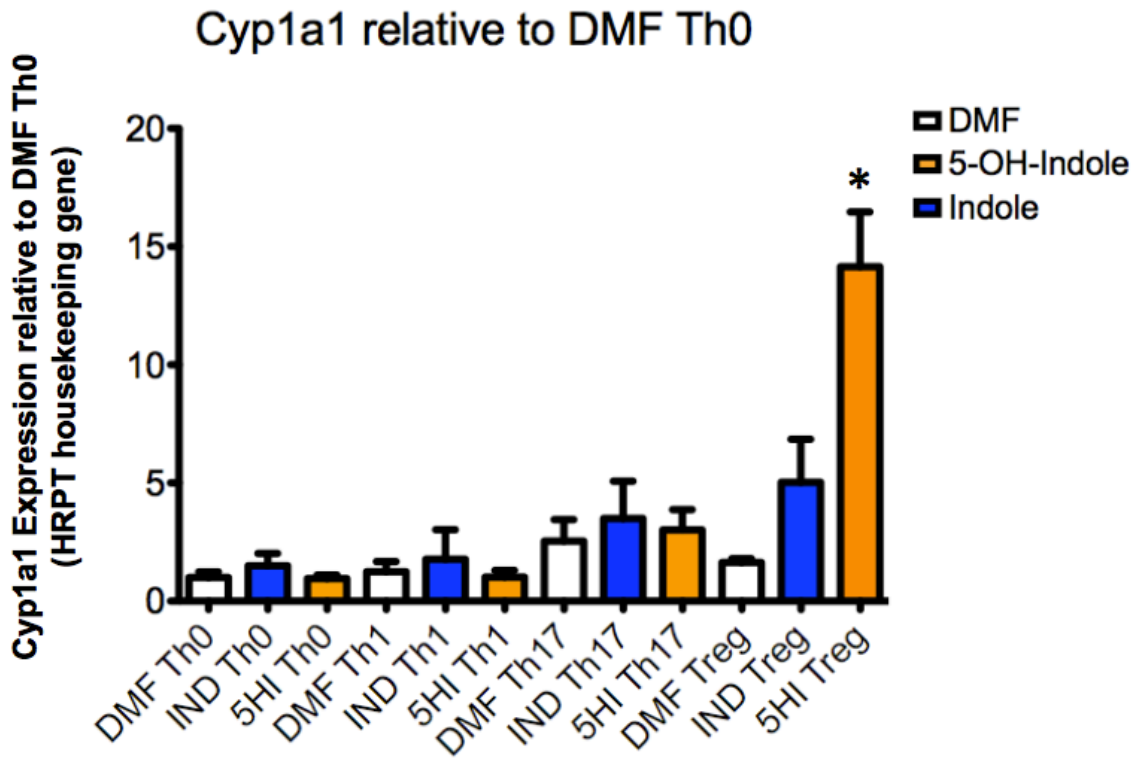

Figure 16. 5-Hydroxyindole augments cyp1a1 transcript levels.

Wild-type CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th0-, Th1-, Th17- or Treg- skew cytokines and 25 μM 5-hydroxyindole, 1 mM indole, or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin, followed by RNA isolation. Isolated RNA was run with one-step qPCR for Cyp1a1 and hypoxanthine guanine phosphoribosyl transferase (HRPT). * denotes $p<0.05$ for one-way ANOVA followed by Dunnet's comparison test to DMF solvent control.

A potential target for metabolic control of T cell differentiation is the molecular target of rapamycin (mTOR). mTOR inhibition by rapamycin has been shown to not only reciprocally regulate Treg and Th17 differentiation but also increase CD62L expression, and we verified these results in both wild-type and AhR knockout mice (fig. 13, 14). Since indole promoted CD62L expression independent of AhR, we investigated the ability of indole to inhibit mTOR. We found that indole inhibited phosphorylation of S6 during T cell stimulation, suggesting inhibition of mTOR activity (fig. 17). This data supports that indole also modulates host cell mTOR signaling, but the fact that indole has effects independent of AhR and not recapitulated by rapamycin (α4β7 induction in FOXP3+ Tregs) suggests additional signaling pathways are affected by indole during T cell differentiation. Due to 5-hydroxyindole's effects being opposite those of indole, we hypothesized that 5-hydroxyindole could act as an mTOR activator; however, we did not find any effect of 5-hyrdoxyindole on S6 phosphorylation, although we did find that rapamycin could block the pro-inflammatory effects of 5-hydroxyindole (fig. 17).

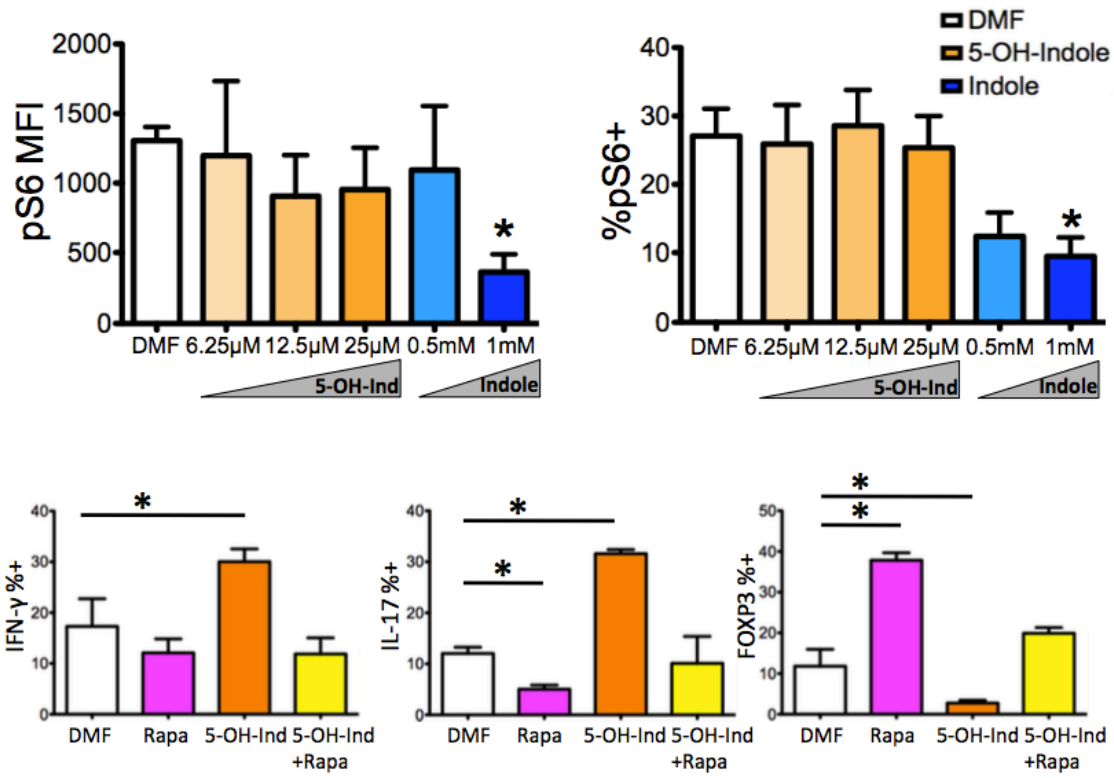

Figure 17. Indole and 5-hydroxyindole interact with mTOR pathway.

Wild-type CD4+ CD25- cells sorted to high purity were cultured with TCR-crosslinking overnight in the presence of indicated concentration of indole, 5-hydroxyindole, or DMF solvent control. At the end of culture, phospho-staining was performed for the S6 ribosomal protein. Both percent positive for pS6 and fluorescence intensity (MFI) of pS6 are shown. Alternatively, CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th1-, Th17- or Treg- skew cytokines and 25 μM 5-hydroxyindole, 1 nM rapamycin, or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug followed by intracellular staining. * denotes $p<0.05$ for one-way ANOVA followed by Dunnet's post-test compared to DMF solvent control.

Additional targets for modulation of Treg and Th17 differentiation include hypoxia-inducible factor-1α (HIF-1α), which can promote proteasomal degradation of FOXP3, promotion of RORγT transcription, and promotion of glycolysis, a hallmark of effector T cells (125). We interrogated this circuit via MG132, a synthetic proteasome inhibitor, 2-deoxyglucose, a glycolysis inhibitor, and qPCR quanitification of HIF-1α expression. Using our standard differentiation assay, we found no appreciable interaction between 5-hydroxyindole and 2-deoxyglucose or MG132 (data not shown); however, we did find an unexpected decrease in HIF-1α expression in T cells cultured with 5-hydroxyindole (fig. 18). The literature is conflicted regarding the role HIF-1α plays during T cell differentiation, with some groups observing that genetic knockout of HIF-1α promotes Treg differentiation and inhibits effector Th17 differentiation (125), while others have found hypoxia-induced HIF-1α stability promotes both Th17 and Treg development (126), and still other labs have described potential inhibition of Th1 differentiation by HIF-1α (127). In agreement with the evidence in the literature for a nuanced role in T cell differentiation, our results suggest that HIF-1α, as an environmental sensor, could work in a context dependent manner in response to the microbiota-derived Trp metabolite 5-hydroxyindole.

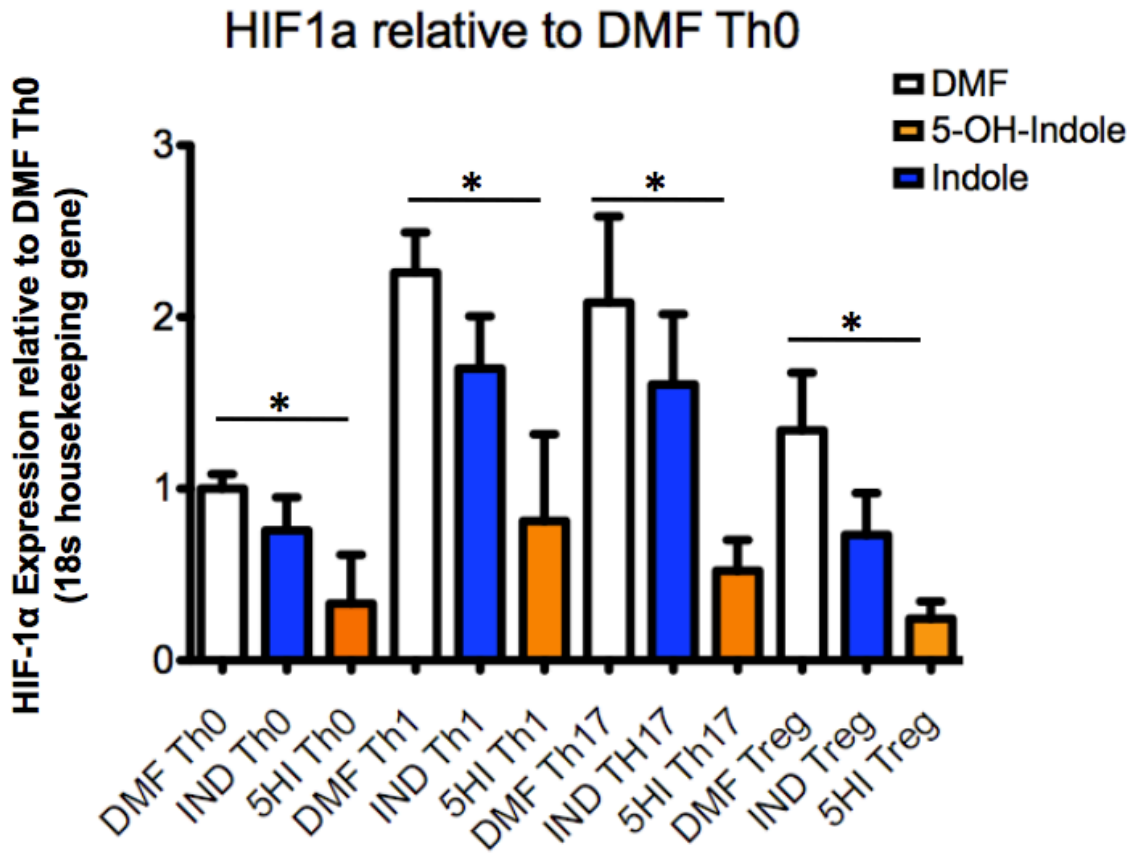

Figure 18. 5-Hydroxyindole decreases transcription of HIF-1α in effector and regulatory T cell skew conditions.

Wild-type CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th0-, Th1-, Th17- or Treg- skew cytokines and 25 μM 5-hydroxyindole, 1 mM indole, or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin, followed by RNA isolation. cDNA from the isolated RNA was run in qPCR for HIF-1α and 18s ribosomal protein. * denotes $p<0.05$ for one-way ANOVA followed by Dunnet's post-test.

Mechanism underlying the effect of butyrate on T cells

In contrast to the emerging immunomodulatory properties of Trp metabolites, which have very little established mechanistic underpinnings, SCFAs have been studied for a relatively long time and the mechanism of host cell signaling is more fully understood. For this reason, we were able to conduct a focused interrogation of the mechanism behind SCFA modulation of T cell differentiation.

Two major pathways of SCFA signaling are currently known: G-protein coupled receptor (GPR) activation and histone deacetylase (HDAC) inhibtion. Most GPRs that recognize SCFAs signal through the G(i) pathway, decreasing cyclic AMP and protein kinase A activity, and researchers have used Pertussis Toxin, an inhibitor of the G(i) pathway, in order to verify the importance of GPR signaling for mediating SCFA effects on cellular behavior (73). We found that pertussis toxin did not affect our results concerning SCFA modulation of T cell differentiation (data not shown).

Indeed, the literature suggests that most effects of SCFAs on T cells are mediated through HDAC inhibition, and we used a screen of several synthetic HDAC inhibitors to try and recapitulate the effects of SCFAs on T cell differentiation. A synthetic class II HDAC inhibitor, as well as a pan HDAC inhibitor, trichostatin A (TSA), did not have effects similar to those seen with the SCFAs (data not shown); however, a class I HADC inhibitor did in fact have strikingly similar effects on T cell differentiation (figs. 19,20,21).

Of note, whereas other researchers have used the pan-HDAC inhibitor TSA to support the role of SCFAs as a strictly anti-inflammatory signal during T cell differentiation (82) or even a promoter of pro-inflammatory Th17 and Th1 differentiation (83), we found that further refining the analysis by using a class I HDAC inhibitor more closely related to the observed HDAC inhibition of SCFAs (62), we see a more complete picture, in which a low dose of Butyrate or a class I HDAC inhibitor can promote effector Th1, Th17 and regulatory T cells, and a high dose of Butyrate or a class I HDAC inhibitor completely blocks all differentiation except towards the Th1 lineage.

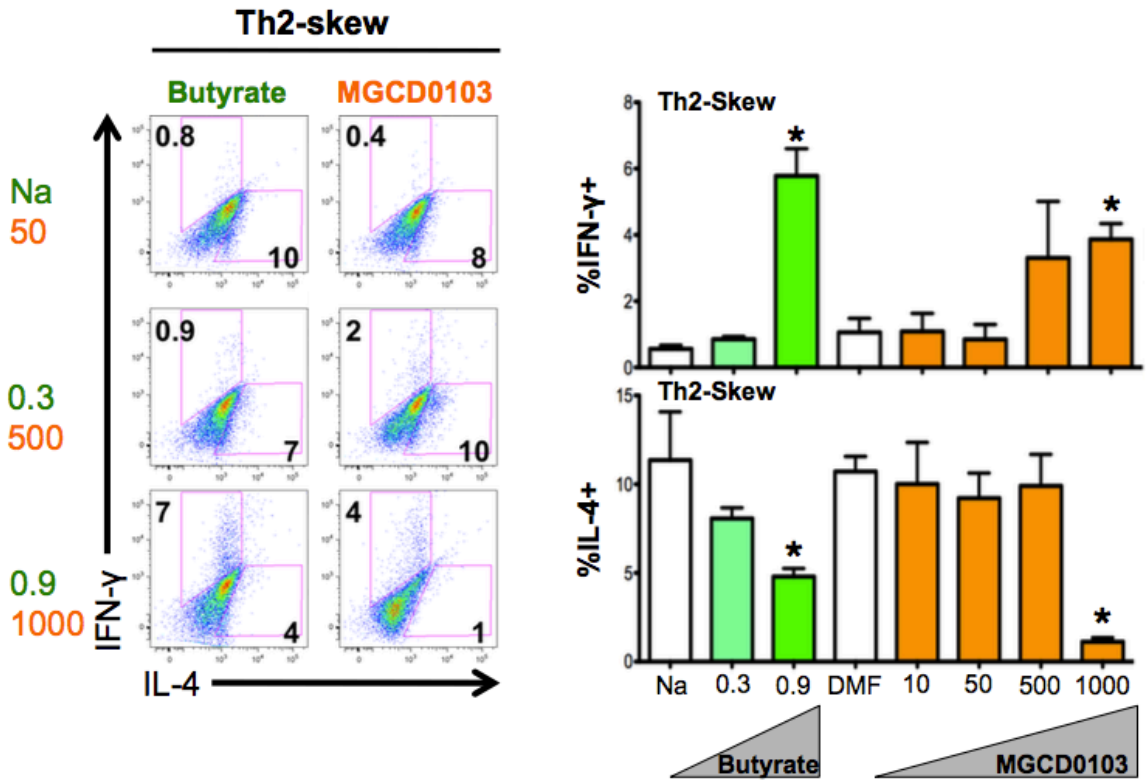

Figure 19. Synthetic class I HDAC inhibitor MGCD0103 affects Th2 differentiation similarly to butyrate.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th2-skew cytokines and butyrate, NaCl control, MGCD0103, or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining. Representative flow data is shown with aggregate bar graphs. Green corresponds to butyrate (dose in mM) and orange corresponds to the synthetic Class I HDAC inhibitor MGCD0103 (dose in nM). * denotes $p<0.05$ for one-way ANOVA followed by Dunnet's comparison test to respective DMF solvent or NaCl control.

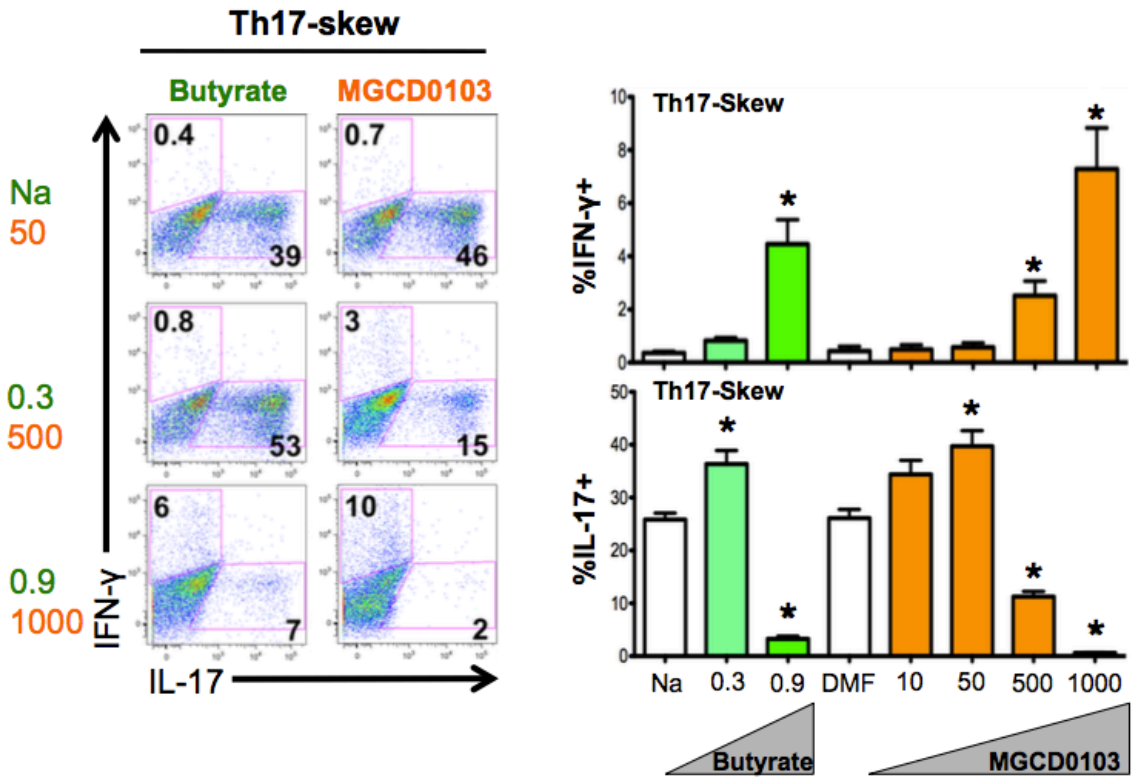

Figure 20. Synthetic class I HDAC inhibitor MGCD0103 affects Th17 differentiation similarly to butyrate.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th17-skew cytokines and butyrate, NaCl control, MGCD0103, or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining. . Representative flow data is shown with aggregate bar graphs. Green corresponds to butyrate (dose in mM) and orange corresponds to the synthetic Class I HDAC inhibitor MGCD0103 (dose in nM). *denotes $p<0.05$ as determined by one-way ANOVA followed by Dunnet's comparison test to respective DMF solvent or NaCl control.

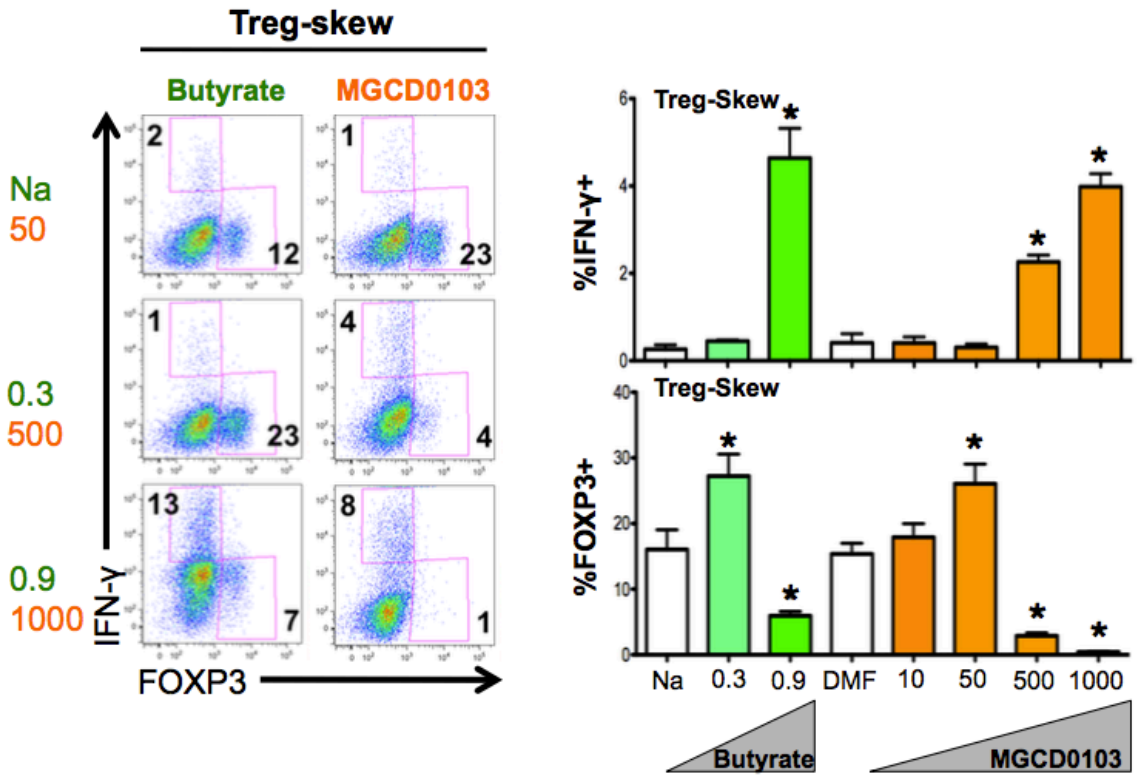

Figure 21. Synthetic class I HDAC inhibitor MGCD0103 affects Treg differentiation similarly to butyrate.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Treg-skew cytokines and butyrate, NaCl control, MGCD0103, or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining. Representative flow data is shown with aggregate bar graphs. Green corresponds to butyrate (dose in mM) and orange corresponds to the synthetic Class I HDAC inhibitor MGCD0103 (dose in nM). *denotes $p<0.05$ as determined by one-way ANOVA followed by Dunnet's comparison test to respective DMF solvent or NaCl control.

Combinations of metabolites produce novel outcomes during T cell differentiation

These surprising findings when using only one metabolite at a time during T cell differentiation made us wonder, what happens in a more physiologically relevant situation where T cells might receive multiple metabolite signals simultaneously? In order to address this question, we performed interaction studies with the SCFAs and Trp metabolites.

Within the Trp metabolites, there is an interesting relationship that emerged. When an active dose of indole and 5-hydroxyindole were added to T cell cultures, we found that Th1, Th17, and Treg differentiation levels were at baseline (fig. 22). This suggests that these two Trp metabolites act as a rheostat, such that the ratio of indole:5-hydroxyindole determines the outcome of T cell differentiation. Specifically, a relatively high indole signal promotes Treg differentiation, a relatively high 5-hydroxyindole signal promotes Th17 and Th1 differentiation, and equal signal from both metabolites are cancelled out.

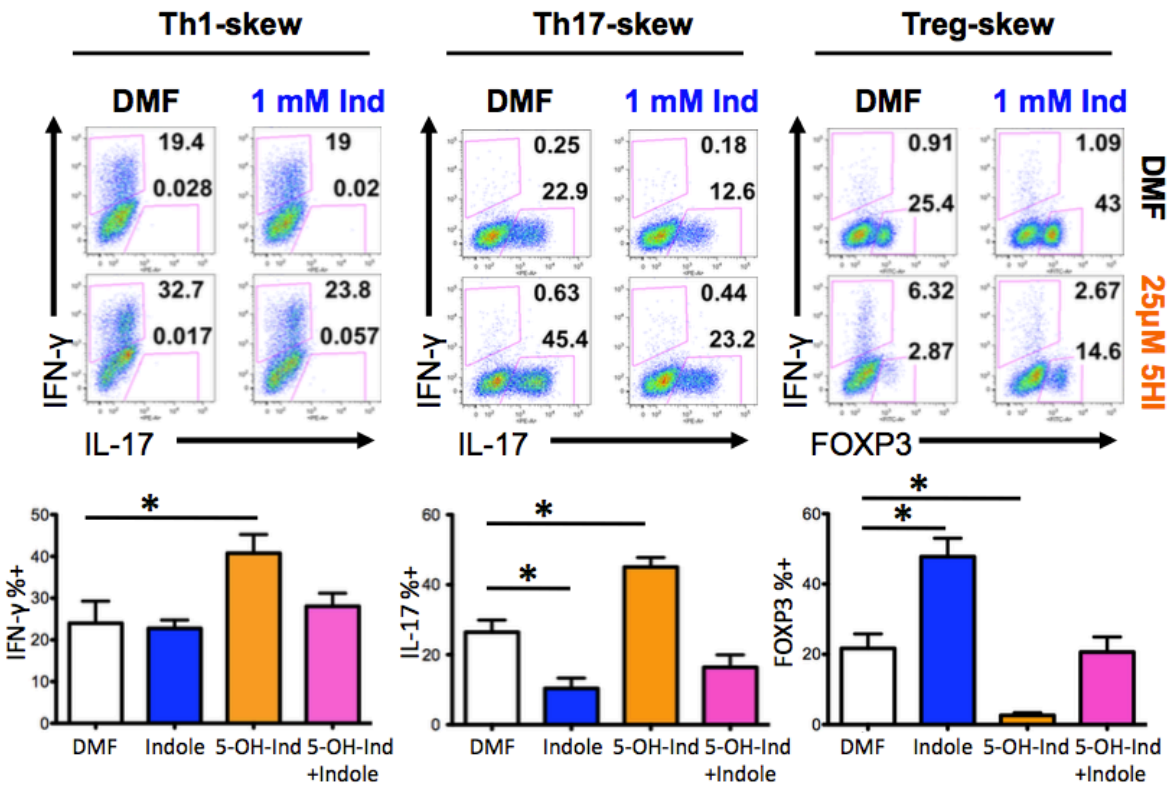

Figure 22. Indole and 5-hydroxyindole interact as a rheostat during T cell differentiation.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th1-, Th17-, or Treg- skew cytokines and indole, 5-hydroxyindole, or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining. * denotes $p<0.05$ as determined by one-way ANOVA followed by Dunnet's post-test.

We also compared Trp metabolite interaction with SCFAs. We found that 5-hydroxyindole promoted the pro-inflammatory Th1 and Th17 augmentation effect of low dose butyrate (figs. 23,24) and blocked the anti-inflammatory Treg augmentation effect of low dose butyrate (fig. 25). However, 5-hydroxyindole could not overcome Th17 inhibition by high dose butyrate (fig. 24). In contrast, indole was able to promote the anti-inflammatory Treg augmentation effect of low dose butyrate (fig. 25) and block the pro-inflammatory Th1 and Th17 augmentation effect of low dose butyrate (figs. 23,24). Indole also blocked Th1 augmentation by high dose butyrate (fig. 23) but could not overcome Treg inhibition by high dose butyrate (fig. 25).

Despite the fact that two of the metabolites—low dose butyrate and 5-hydroxyIndole—augment Th1 and Th17 differentiation, when these two are combined with indole, we find that Th1 and Th17 differentiation is at baseline level, suggesting nonlinear interaction between these three metabolites during T cell differentiation towards pro-inflammatory lineages (figs. 23,24). Additionally, as we would expect from the fact that both indole and low dose butyrate promote Treg differentiation, we see that in the presence of all three metabolites, there is augmented Treg differentiation compared to baseline (fig. 25).

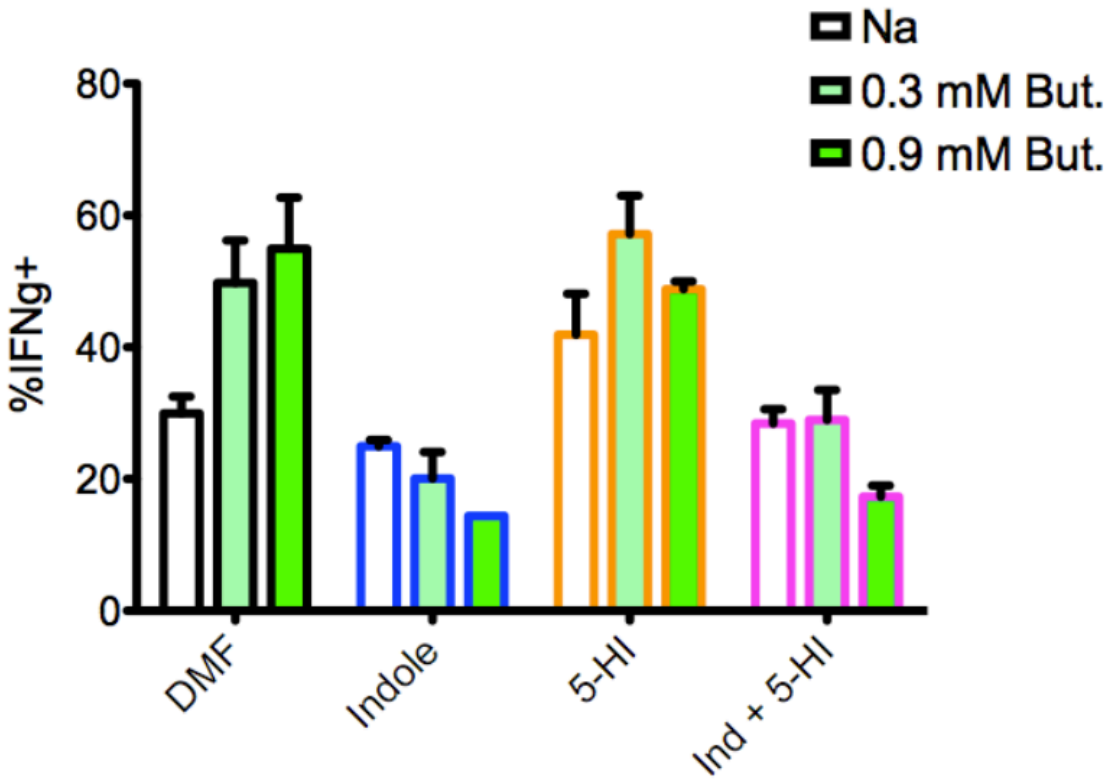

Figure 23. Butyrate, indole and 5-hydroxyindole interact during Th1 differentiation.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th1-skew cytokines; butyrate or NaCl control; and indole, 5-hydroxyindole, or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining.

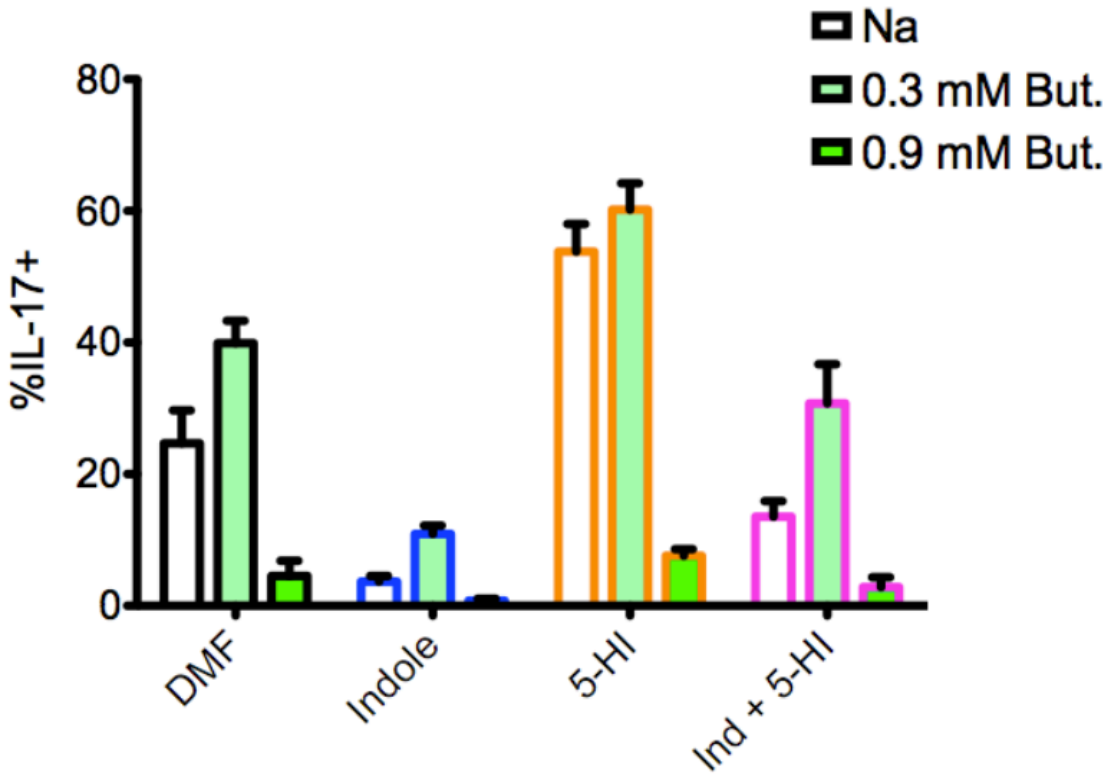

Figure 24. Butyrate, indole and 5-hydroxyindole interact during Th17 differentiation.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th17-skew cytokines; butyrate or NaCl control; and indole, 5-hydroxyindole, or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining.

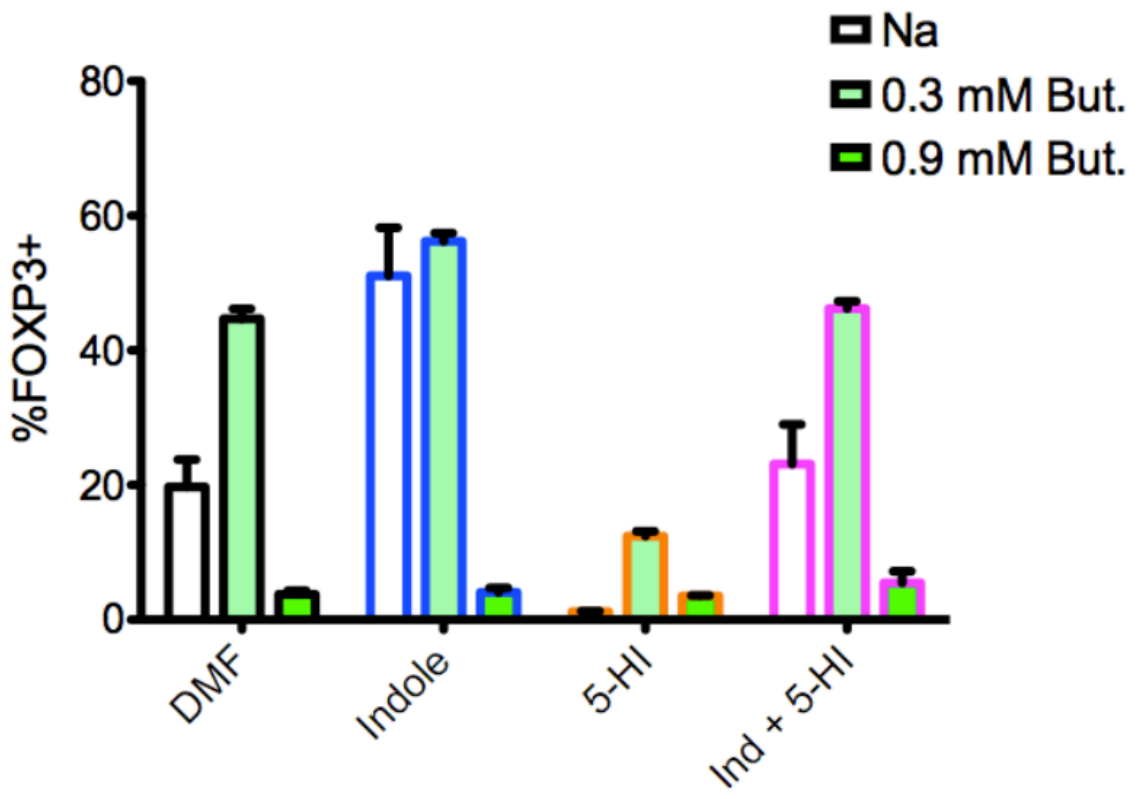

Figure 25. Butyrate, indole and 5-hydroxyindole interact during Treg differentiation.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Treg-skew cytokines; butyrate or NaCl control; and indole, 5-hydroxyindole, or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining.

Human T cell differentiation is modulated by Trp metabolites

We verified key results seen with mouse T cells in a human T cell differentiation assay. Some general observations about the difference between human and mouse T cell culture are warranted, because they play a role in how we will interpret the data.

In terms of cytokine factors necessary, Treg differentiation *in vitro* is by and large very similar between mice and humans (specifically, TGF-β and IL-2). The only major difference between mice and human Treg differentiation is the larger amount of variability in baseline Treg induction in T cells from separate human donors. This variability can be accounted for by analyzing our data in terms of paired observations for each donor sample, as opposed to an aggregate of unpaired observations that is typically used in mouse studies (128).

Th17 differentiation has been reported in the literature to require different cytokine signals in human cells compared to mouse cells. Whereas in mouse cells, Th17 differentiation can be consistently induced by TGF-β, IL-6, and IL-23; human Th17 differentiation has been reported to require TGF-β, IL-1β, IL-6 and IL-23. Furthermore, the level of Th17 differentiation is usually very low in human cells, usually averaging around 1-2% IL-17+ at the end of a 7-10 differentiation culture (128-130). We found that these results were true in our hands as well, and the low yield of Th17 differentiation could play a role in the results we obtained, particularly in terms of indole's lack of effect on human Th17 differentiation.

From six donors, we found a significant augmentation of Treg differentiation by indole (fig. 26). Th17 differentiation was not significantly affected by indole treatment, and this is likely a result

of having a low baseline level (fig. 27). On the other hand 5-hydroxyindole significantly inhibited Treg differentiation and significantly augmented Th17 differentiation (figs. 26, 27). Among the SCFAs, propionate significantly augmented Treg differentiation, high dose (0.9 mM) butyrate inhibited Treg differentiation, and acetate and low dose (0.3 mM) butyrate had no significant effect (fig. 28). Surprisingly, we found that Propionate also inhibited Th17 differentiation, in contrast to results in mouse T cells that showed propionate and other SCFAs increase Th17 differentiation (fig. 29).

Notably, our baseline Th17 differentiation with NaCl control is higher than our DMF control, and reports have been published of NaCl promoting Th17 differentiation(131). Although this study found 40mM NaCl was required to augment Th17 differentiation *in vitro*, it was performed on mouse cells, so it could be the case that human cells are more sensitive to the Th17 promotion of NaCl. Regardless, these results suggest that SCFAs do not promote Th17 differentiation in human cells.

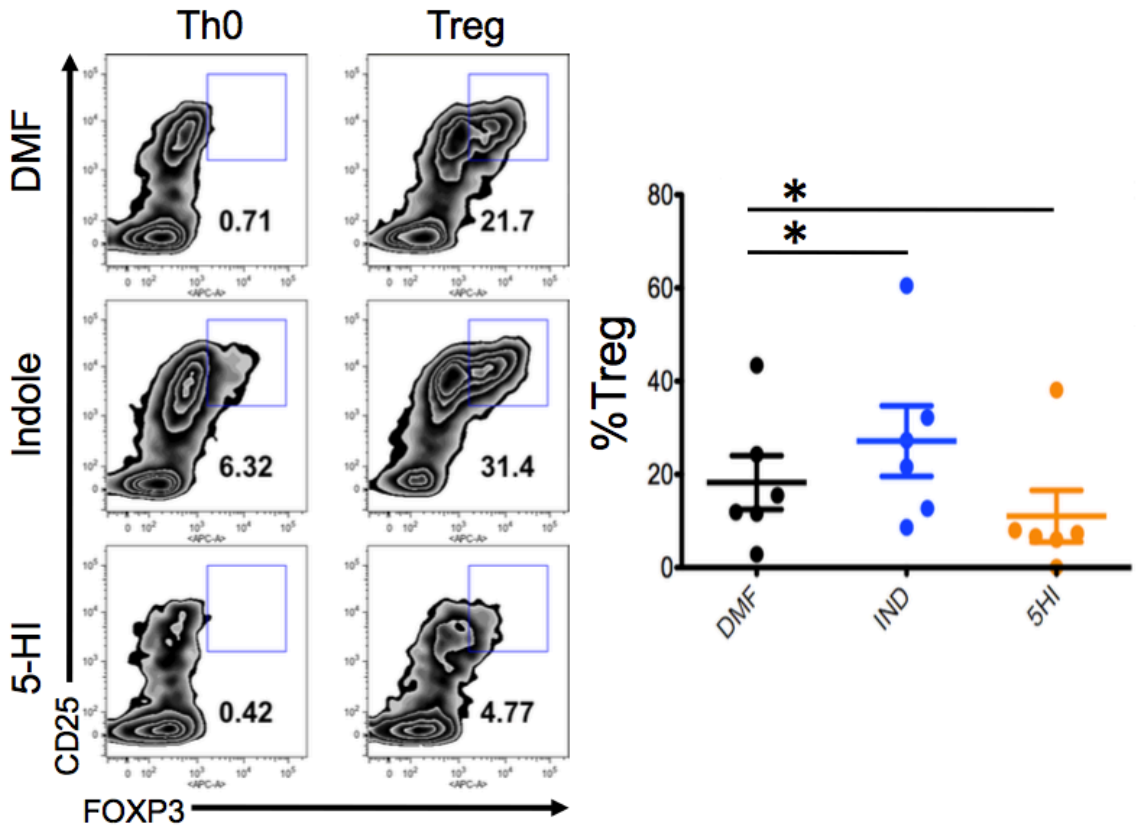

Figure 26. Human Treg differentiation is regulated by microbiota-derived Trp metabolites.

Human naïve CD4+ CD45RO- T cells were isolated to high purity from enriched blood of a single donor for each experiment. Cells were cultured for 7-10 days with TCR crosslinking in the presence of Treg-skew cytokines and indole, 5-hydroxyindole, or DMF solvent control. * denotes $p<0.05$ for paired t test.

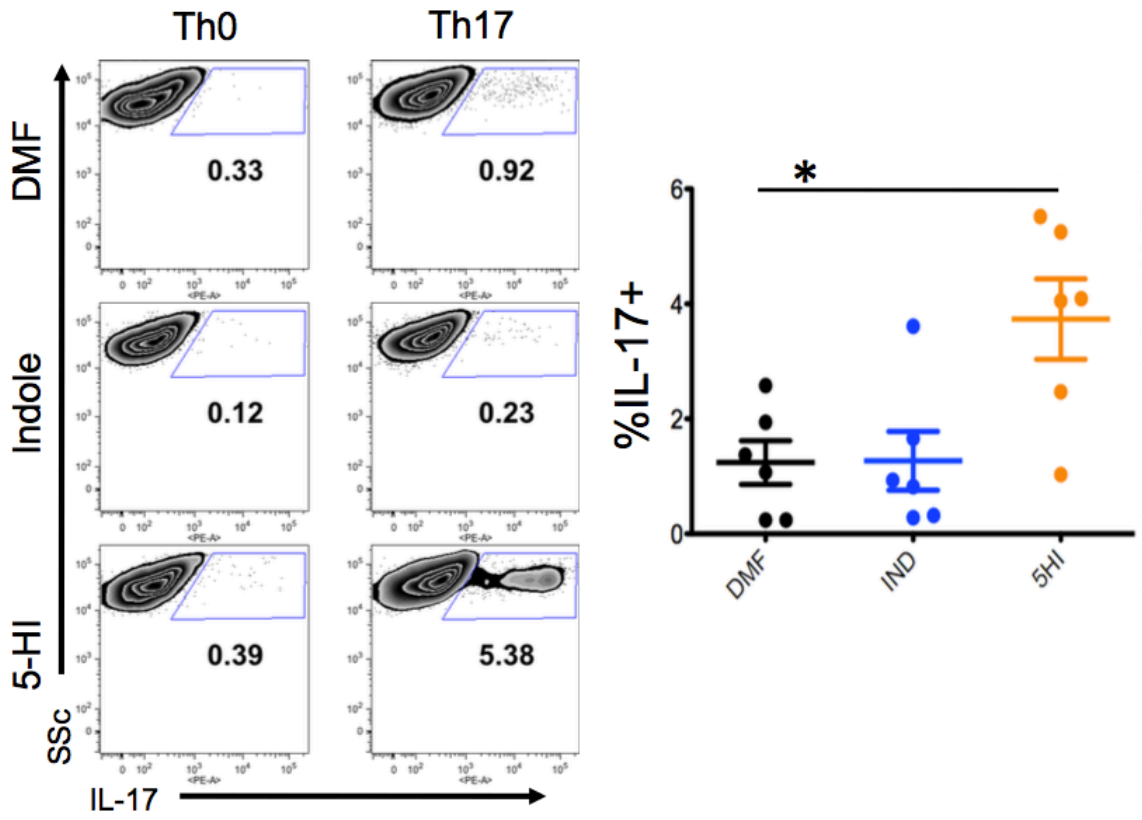

Figure 27. Human Th17 differentiation is regulated by microbiota-derived Trp metabolite.

Human naïve CD4+ CD45RO- T cells were isolated to high purity from enriched blood of a single donor for each experiment. Cells were cultured for 7-10 days with TCR crosslinking in the presence of Th17-skew cytokines and indole, 5-hydroxyindole, or DMF solvent control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining. * denotes $p<0.05$ for paired t test.

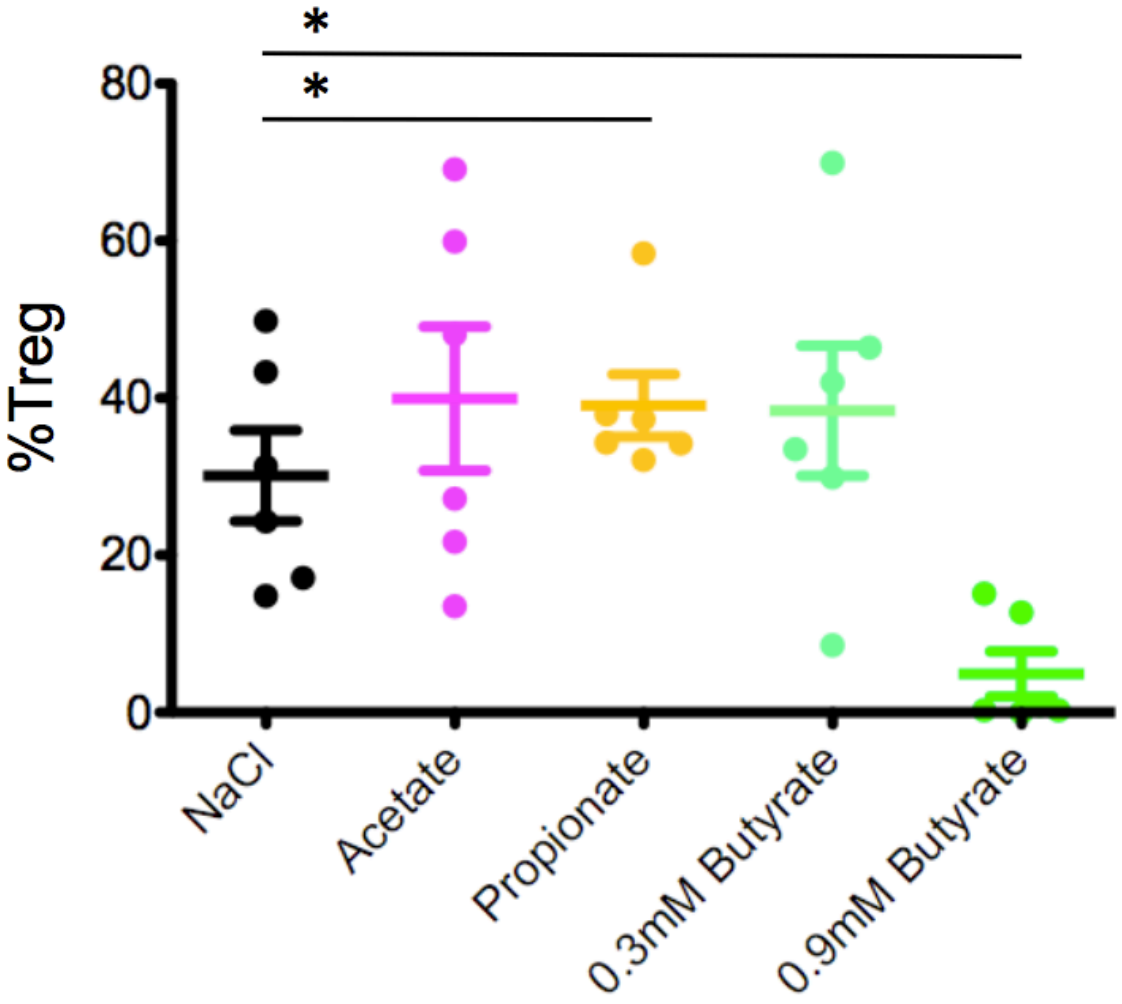

Figure 28. Human Treg differentiation is regulated by microbiota-derived SCFA.

Human naïve CD4+ CD45RO- T cells were isolated to high purity from enriched blood of a single donor for each experiment. Cells were cultured for 7-10 days with TCR crosslinking in the presence of Treg-skew cytokines and acetate, propionate, butyrate, or NaCl control. * denotes $p<0.05$ for paired t test.

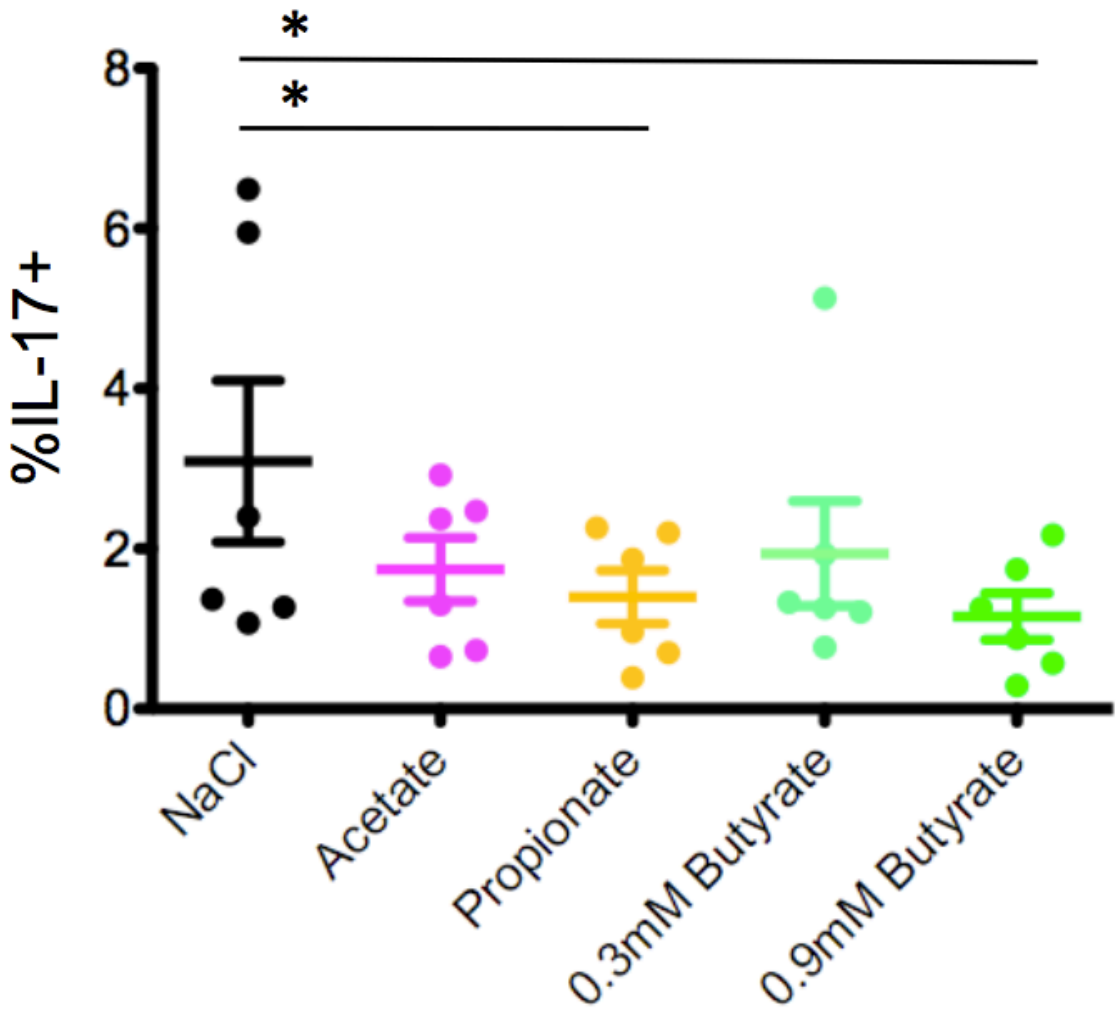

Figure 29. Human Th17 differentiation is regulated by microbiota-derived SCFA.

Human naïve CD4+ CD45RO- T cells were isolated to high purity from enriched blood of a single donor for each experiment. Cells were cultured for 7-10 days with TCR crosslinking in the presence of Th17-skew cytokines and acetate, propionate, butyrate, or NaCl control. For the last 4 hours of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug, followed by intracellular staining. * denotes $p<0.05$ for paired t test.

Discussion

We found that Trp metabolites are generally able to modulate T cell differentiation just as SCFA metabolites have been demonstrated to, but the specific effects between and within these classes are diverse. Indole and 5-hydroxyindole stood out among the Trp metabolites as having the most robust effects on T cell differentiation, and these two metabolites had contrasting effects. Specifically, indole promotes Treg and inhibits Th17 differentiation, while 5-hydroxyindole promotes Th1 and Th17 and inhibits Treg differentiation. The SCFAs had effects that can be broken up in to two groups: 1) low dose butyrate, acetate, and propionate 2) high dose butyrate. Group 1 promoted effector Th1 and Th17 differentiation as well as Treg differentiation. Group 2 inhibited all lineage differentiation except Th1 and interestingly was able to induce IFN-γ expression in Th0-, Th2-, Th17-, and Treg- skew conditions.

The iTregs produced in our differentiation assays had altered homing marker and cytokine expression, which has also been observed for other Treg-promoting compounds like retinoic acid and rapamycin. Specifically, we found that indole-conditioned iTregs had increased gut and lymph node homing-marker expression as well as two-fold increased TGF-β and essentially no IL-10 production. Butyrate-conditioned iTregs did not have altered homing marker expression, but did have a roughly two-fold increase in IL-10 production. The fact that microbiota-derived metabolites condition specific phenotypes of iTreg cells is very intriguing and adds an additional layer to microbiota regulation of the host immune response. The particular metabolite signals could be better suited to alleviate specific types of inflammation, and further research is necessary to establish whether or not specific metabolite classes can have varied therapeutic efficacy for different disease models.

In the GI tract, we might expect these metabolites to act in concert on T cell differentiation, and we found that the combination of metabolites can produce unexpected results. In general, we saw that the combination of indole and 5-hydroxyindole act as a rheostat, such that the ratio of indole to 5-hydroxyindole will determine whether anti- or pro- inflammatory T cell differentiation is promoted. Additionally, we found that indole and 5-hydroxyindole promote the anti- and pro-inflammatory properties of butyrate, respectively, and we have seen this is also true for the other SCFAs propionate and acetate (data not shown). The combination of indole, 5-hydroxyindole, and butyrate produced a surprising relationship wherein only anti-inflammatory Treg differentiation is promoted above baseline and Th1 and Th17 differentiation remain at baseline. From simply studying the interaction of three metabolites with unique effects on T cell differentiation, a non-linear relationship has emerged that could explain how a microbiota net promotion of T cell tolerance can occur even when metabolites with singular pro-inflammatory properties are being produced. These results suggest that studying microbiota metabolite signals in concert could provide a better understanding of the homeostatic relationship between host and microbiota.

Mechanistically, SCFA effects appear to be mediated by histone deacetylase inhibition, a result recapitulated by numerous groups' published work. 5-Hydroxyindole's effect on T cell differentiation appeared to be partially independent of the aryl hydrocarbon receptor (AhR), as we only saw an attenuated promotion of Th17 differentiation in AhR knockout T cells. Indole's effect on T cell differentiation is dependent on AhR, but its effects on homing-marker expression are independent of AhR. Interestingly, we determined that indole downregulates phosphorylation

of the ribosomal S6 protein, suggesting that indole acts as an mTOR inhibitor, and rapamycin, the canonical mTOR inhibitor promotes lymph-node homing markers similarly to what we see with indole. However, we still do not have a mechanistic explanation for increased gut-homing marker expression observed with indole conditioning. As a next step toward mechanistic pathways affected by the Trp metabolites, we are currently executing microarray studies of purified FOXP3-GFP+ indole-conditioned iTregs and IL-17-GFP+ 5-hydroxyindole-conditioned Th17 cells. This transcriptomic approach will be useful to direct future studies in the lab.

Importantly, we found that human CD4+ T cell differentiation is also modulated by microbiota-derived metabolites. Treg differentiation was promoted by indole and propionate and inhibited by 5-hydroxyindole and high dose butyrate. Th17 differentiation was very low at baseline, but 5-hydroxyindole was a potent promoter of IL-17 expression. On the other hand Th17 differentiation was not affected by indole and actually inhibited by propionate, in contrast to our results in mouse cells. This result with propionate may actually be a result of NaCl inducing Th17 differentiation in our baseline condition, as described in the results section. However, the results support further study of metabolite production from the microbiota as a therapeutic and/or diagnostic modality in clinical practice.

CHAPTER III

MICROBIOTA-DERIVED METABOLITES MODULATE T CELL LINEAGE CHOICE IN VIVO

Overview

The behavior of CD4+ T helper cells as orchestrator of the adaptive immune response requires integration of a complex array of environmental signals. Thus, studies of T cell function *in vitro* are intirinsically limited in their applicability to physiologic settings due to our inability to recreate the complex environment of a living multicellular organism in a petri dish. We proceeded to test our major observations about microbiota-derived metabolites with to experimental *in vivo* systems and found:

1. In an acute T cell activation model, indole and butyrate promote FOXP3 expression while indole blocks the promotion of IFN-γ expression by butyrate.

2. In a chronic T cell transfer colitis model, indole and 5-hydroxyindole have a lasting effect on T cells conditioned *in vitro* that results in alleviated and exacerbated colitis, respectively.

Rationale

Our results from chapter 1 suggest that microbiota metabolites have effects on T cells while they are exposed to metabolite, but an additional question is whether or not the effect of metabolite conditioning can last beyond the time period of exposure. This is particularly relevant when considering these metabolites as conditioning agents for cellular therapeutic preparation *in vitro* prior to adoptive transfer. We made use of two models of T cell function that have been used by

other groups studying short-chain fatty acid modulation of T cell differentiation. One model is an acute activation model that allows us to verify findings from the *in vitro* differentiation assay, while the second model allows us to address the question of the durability of metabolite conditioning.

Results

Acute T cell activation in vivo recapitulates indole and butyrate interaction

We used an *in vivo* model of acute T cell activation via co-administration of the anti-CD3 antibody clone 145-2C11 and butyrate, indole or a combination of the two metabolites. Administration of the T cell activating anti-CD3 antibody caused increased IFN-γ expression in T cells, which was augmented by co-administration with butyrate and brought down to baseline with co-administration of a combination of butyrate and indole (fig. 30). Additionally, FOXP3 expression was only significantly increased compared to a vehicle control when the T cell activating anti-CD3 antibody was co-administered with indole alone or a combination of indole and butyrate (fig. 31). Importantly, we found that these effects were only present in the CD44lo CD4+ T cell population and not the CD44hi CD4+ memory T cell population. These observations further support that indole and butyrate have antagonistic and complimentary effects on the differentiation of naïve T cells into effector and regulatory lineages, respectively.

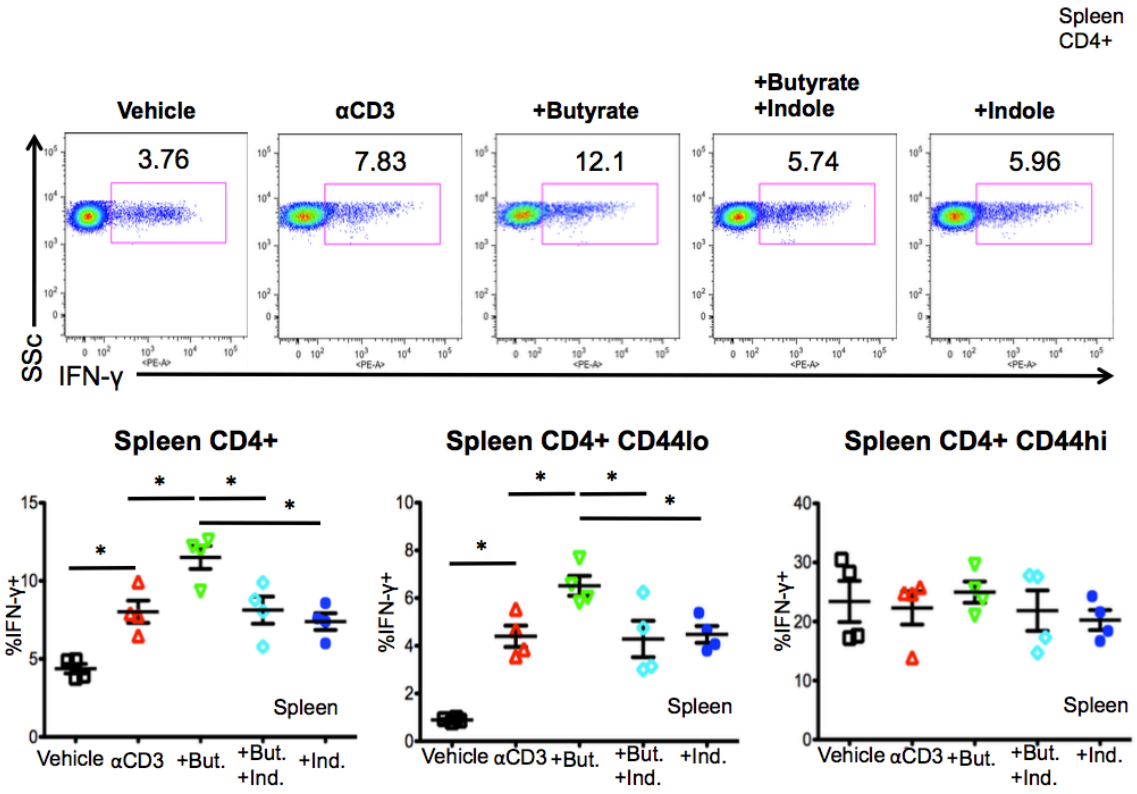

Figure 30. Butyrate and indole interact to maintain baseline IFN-γ *in vivo* during acute T cell activation.

Splenocytes from mice injected intraperitoneally with vehicle or αCD3 with indole, butyrate, a combination of the two, or DMF solvent control were stimulated with PMA and ionomycin in the presence of golgi plug followed by surface and intracellular staining. * denotes $p<0.05$ for one-way ANOVA followed by Newman-Keuls post test.

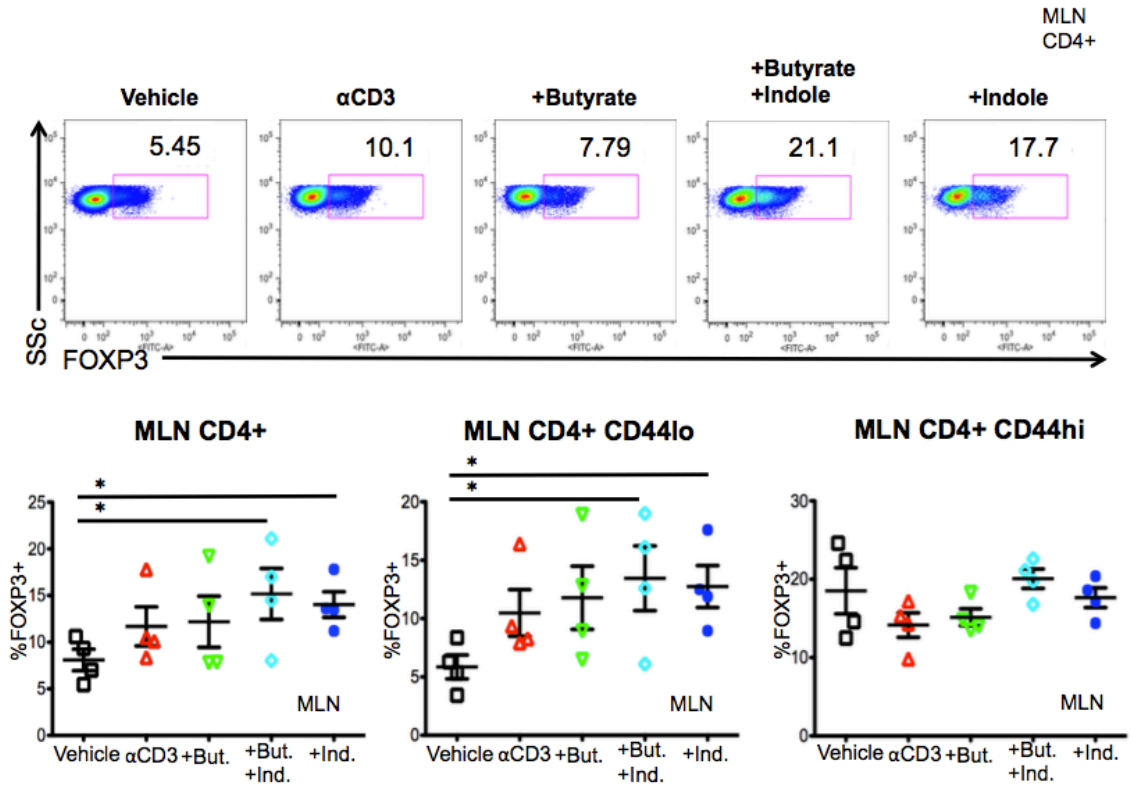

Figure 31. Butyrate and indole interact to promote FOXP3 *in vivo* during acute T cell activation.

Mesenteric lymph node cells from mice injected intraperitoneally with vehicle or αCD3 with indole, butyrate, a combination of the two, or DMF solvent control were stimulated with PMA and ionomycin in the presence of golgi plug followed by surface and intracellular staining. * denotes $p<0.05$ for one-way ANOVA followed by Newman-Keuls post test.

Th17 cell transfer colitis recapitulates indole and 5-hydroxyindole effects on T cell function

We attempted to use this same acute T cell activation model to verify the effects of 5-hydroxyIndole on T cell activation and differentiation, but we found no effect at multiple doses (data not shown). Instead, we focused on a modified model of T cell transfer colitis also used by *Park et al* (83). This model uses adaptive immune cell deficient $RAG^{-/-}$ mice as recipients of 10^6 non-purified cells from a culture of naïve wild-type T cells skewed towards Th17. As the cells proliferate *in vivo*, chronic colitis develops.

Importantly, *Park et al* (83) observed that Th17 cultures conditioned with SCFA had a higher proportion of IL-17+ cells compared to solvent control cultures; however, the SCFA conditioned cultures caused a less severe colitis, suggesting that not only is the proportion of IL-17+ cells in the transferred population important but metabolite conditioning is also a critical and independent factor. With these aspects of the model in consideration, we cultured naïve wild-type CD4+ CD25- T cells towards Th17 in the presence of DMF solvent control, indole, or 5-hydroxyindole.

The Th17 cultures we introduced into the $RAG1^{-/-}$ mice were representative of what we typically see in experiments. Specifically, baseline Th17 differentiation with DMF solvent control was 9%, while indole had a marked inhibition to 2.5%, and 5-hydroxyindole increased the Th17 differentiation by roughly 3-fold to 28% (see methods). After cell transfer, we recorded weight weekly until mice started losing weight, at which point we recorded weight every other day. Additionally, we harvested spleen, mesenteric lymph node and distal lymph nodes at day 15 and day 49 of the experiment to look at cell dynamics during the course of colitis.

For both time points, the mesenteric lymph nodes of RAG mice that received T cell transfer had a similar number of CD4+ T cells that was lower than what is seen in wild-type C57Bl/6 mice (fig. 32). We found that after two weeks of expansion, the mesenteric lymph nodes of mice received indole-conditioned Th17 cultures had a significantly increased proportion of FOXP3+ Tregs, whereas the mice receiving DMF solvent control- and 5-hydroxyindole- conditioned Th17 cultures had a similar smaller percentage of Tregs (fig. 33). Additionally, the proportion of IFN-γ+ Th1 and IL-17+ Th17 cells was significantly increased in mice that received either indole- or 5-hydroxyindole- conditioned Th17 cultures compared to mice that received DMF solvent control-conditioned Th17 cultures (fig. 34, 35). However, it is important to note that these results as a whole mean that the ratio of effector Th1 or Th17 cells to Treg cells is higher in mice that received 5-hydroxyindole-conditioned Th17 cultures compared to mice that received either DMF solvent control- or indole- conditioned Th17 cultures (fig. 34, 35).

At the end of seven weeks, we saw that mice that received indole-conditioned Th17 cultures had little weight loss, whereas the mice that received DMF solvent control-conditioned Th17 cultures had a moderate weight loss and mice that received 5-hydroxyindole-conditioned Th17 cultures had the most severe weight loss (fig. 36). Furthermore, we found that Th1, Th17 and Treg cells had increased in the mesenteric lymph nodes of mice that received either DMF solvent control- or 5-hydroxyindole- conditioned Th17 cultures, whereas mice that received indole-conditioned Th17 cultures had a lower amount of Th1 and Th17 cells (fig. 33, 34, 35).

From these results, we propose an explanation such that indole- and 5-hydroxyindole conditioned Th17 cultures initially expand rapidly into the Th1 and Th17 compartments, but

indole-conditioned Th17 cultures also expand rapidly into the Treg compartment, effectively blocking development of severe colitis and resulting in a quiescent T cell population after 7 weeks. DMF solvent control-conditioned Th17 cells do not expand as rapidly as either metabolite-conditioned culture, but they do show expansion by 7 weeks accompanied by colitis progression.

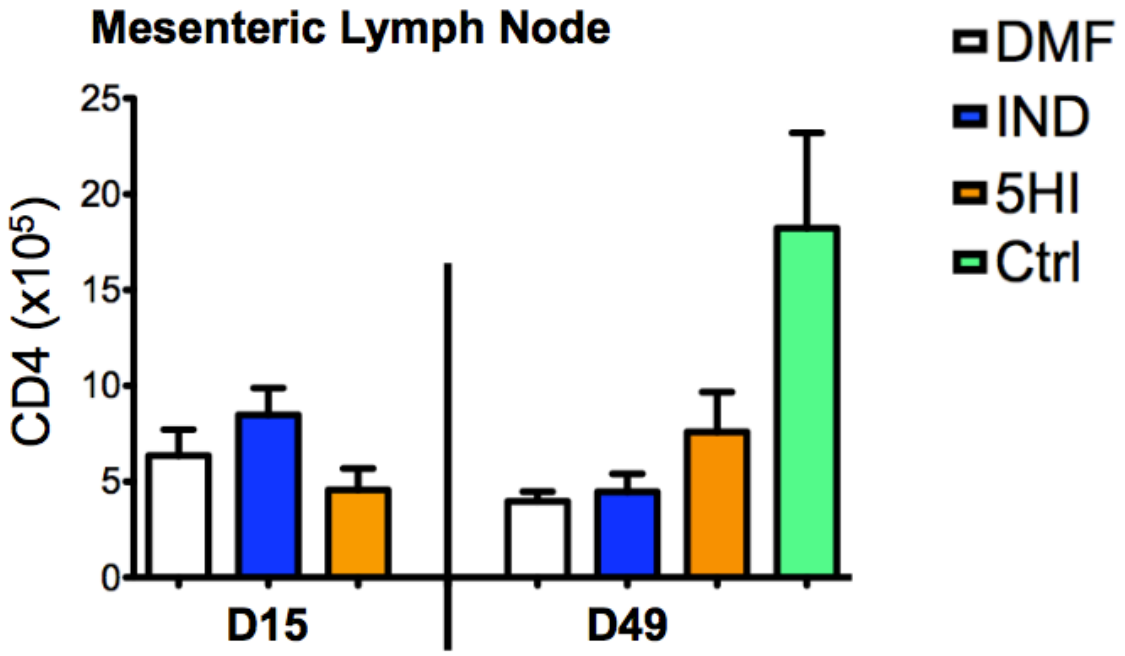

Figure 32. Cellularity in mesenteric lymph node during Th17 transfer colitis.

Mesenteric lymph nodes were harvested at day 15 and day 49 of colitis. Cells from individual mice were counted and stained for CD4, followed by flow cytometry.

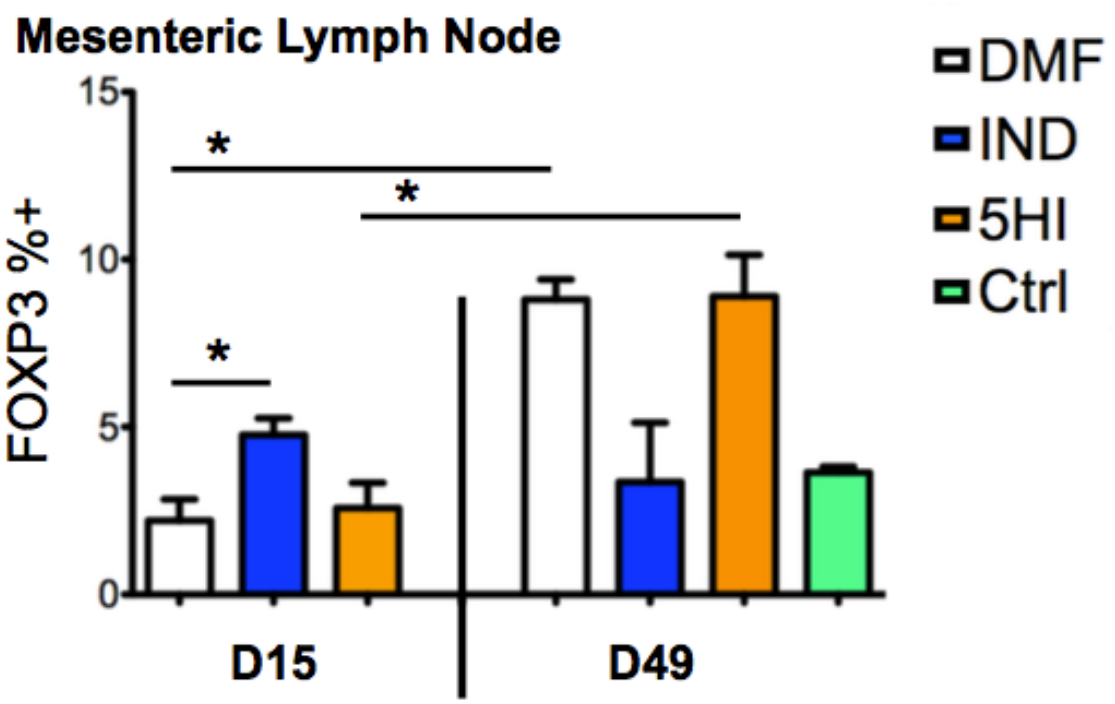

Figure 33. Treg percentage in mesenteric lymph node depends on metabolite conditioning *in vitro* prior to transfer.

Mesenteric lymph nodes were harvested at day 15 and day 49 of colitis. Cells from individual mice (groups are RAG1$^{-/-}$ mice receiving DMF solvent control-, indole- or 5-hydroxyindole-conditioned Th17 cultures and C57Bl/6 wild-type control mice) were stained for CD4 and FOXP3, followed by flow cytometry. Data shown is from gated CD4+ T cells. * denotes $p<0.05$ for student's t test.

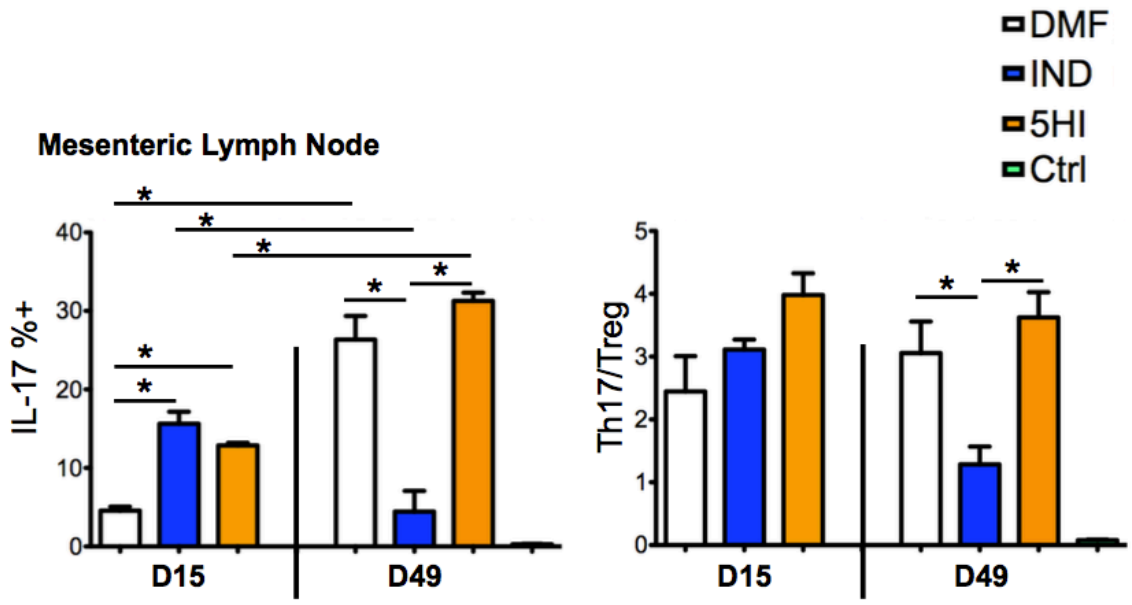

Figure 34. Th17 percentage and ratio to FOXP3+ Tregs in the mesenteric lymph node depends on metabolite conditioning *in vitro* prior to transfer.

Mesenteric lymph nodes were harvested at day 15 and day 49 of colitis. Cells from individual mice (groups are RAG1$^{-/-}$ mice receiving DMF solvent control-, indole- or 5-hydroxyindole-conditioned Th17 cultures and C57Bl/6 wild-type control mice) were stimulated with PMA and ionomycin in the presence of golgi plug followed by intracellular staining. Data shown is from gated CD4+ T cells. * denotes $p<0.05$ for student's t test.

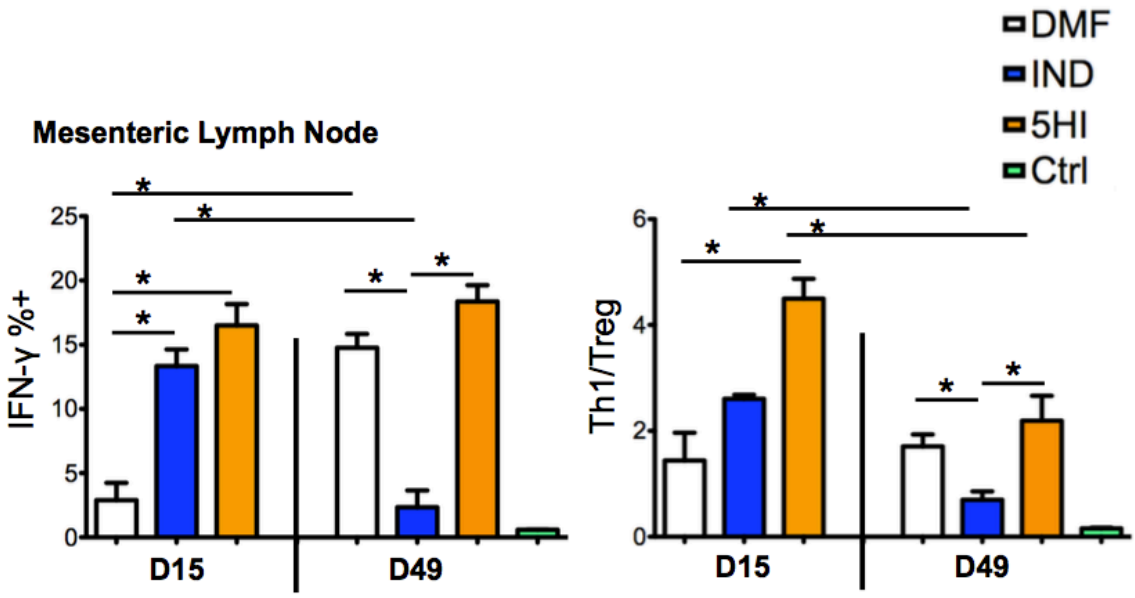

Figure 35. Th1 percentage and ratio to FOXP3+ Tregs in the mesenteric lymph node depends on metabolite conditioning *in vitro* prior to transfer.

Mesenteric lymph nodes were harvested at day 15 and day 49 of colitis. Cells from individual mice (groups are RAG1$^{-/-}$ mice receiving DMF solvent control-, indole- or 5-hydroxyindole-conditioned Th17 cultures and C57Bl/6 wild-type control mice) were stimulated with PMA and ionomycin in the presence of golgi plug followed by intracellular staining. Data shown is from gated CD4+ T cells. * denotes $p<0.05$ for student's t test.

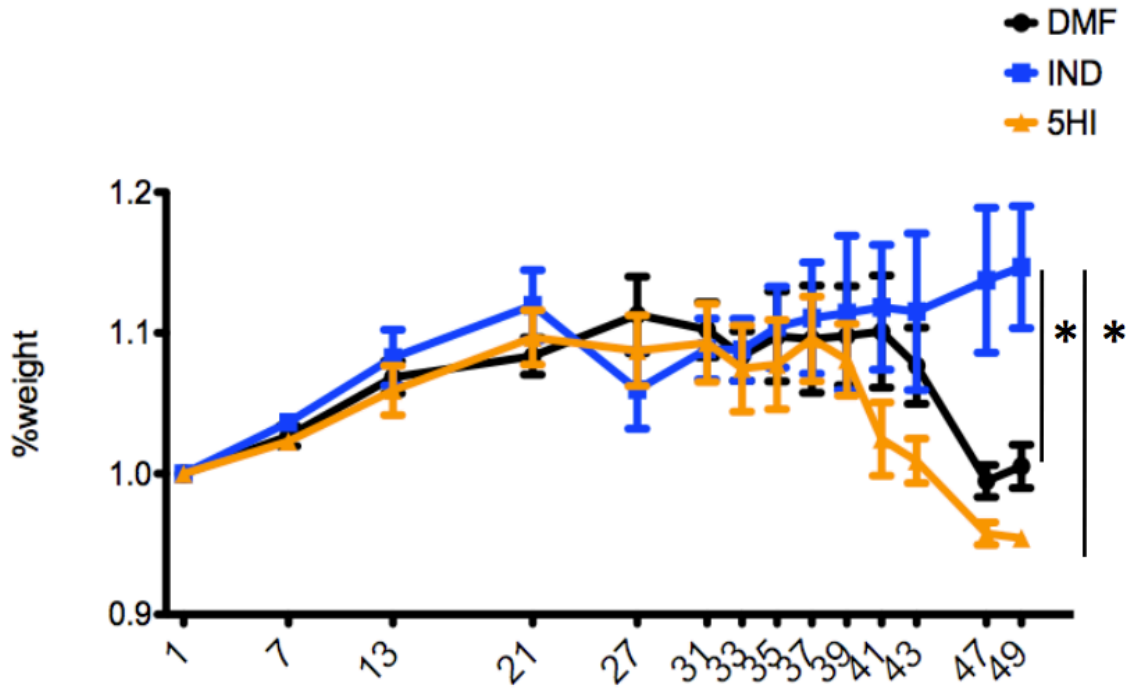

Figure 36. Weight loss during Th17 transfer colitis is dependent on metabolite conditioning *in vitro* prior to transfer.

* denotes $p<0.05$ for two-way ANOVA followed by Bonferonni's post-test.

Discussion

We found that during acute activation of T cells, indole promotes naïve T cell expression of FOXP3 with butyrate and blocks naïve T cell expression of IFN-γ promoted by butyrate. These results suggest that T cell activity in the GI tract, where these metabolites are at their highest concentration, could be shifted towards a pro- or anti-inflammatory response depending on the relative levels of both Trp metabolites and SCFAs.

Additionally, we found that the Trp metabolites indole and 5-hydroxyindole condition T cells during activation in such a way that the expansion of conditioned, activated T cells can shift towards a homeostatic or inflammatory response, respectively, during expansion in a lymphopenic mouse. These results answer an important question about the lasting impact of Trp metabolites on T cell function, considering that these compounds are found predominantly in the GI tract and not in the systemic circulation. On the other hand, SCFAs are not completely removed by first-pass metabolism in the liver and are detectable at relatively high levels in the serum (74), so the SCFAs may not require an effect that lasts beyond exposure in order to affect the host immune response in a systemic manner. That being said, another group has used the same Th17 transfer colitis model and found a similar long-lasting effect of acetate on the expansion of Th17-skewed cultures in a lymphopenic mouse (83).

Whether exposure to Trp metabolites and SCFAs in the GI tract has long lasting or transient effects, our results suggest that metabolite production is an important mechanism behind regulation of immunity by the microbiota. Notably, the concept of T cell plasticity between lineages has gained prominence recently (132). Not only have T cells been demonstrated to

switch lineage transcription factor expression based on real time cues from the environment, but Tregs appear to use effector transcription programs in order to specifically suppress a specific immune response (133). These results suggest an interesting scenario in which unique metabolite signals at a spatially restricted focus of gut-associated lymphoid tissue could alter the quality of incoming effector and regulatory T cell responses.

Furthermore, the ability of these microbiota metabolites to condition cells *in vitro* and confer long-term properties on the conditioned cells as well as their descendants after reintroduction into a host strongly supports the utilization of these compounds for preparation of cellular therapeutics. Recent work has focused on rapamycin and retinoic acid as potential conditioning agents for *in vitro* preparation of a cellular therapeutic (134), but our results in Chapter 1 suggest that use of the microbiota-derived Trp metabolites and SCFAs would provide an expanded array of cellular therapeutic properties such as enhanced gut-homing, lymph node-homing, and cytokine expression abilities.

Importantly, this work has other therapeutic implications besides cellular therapy. The ability to mount an inflammatory response at the mucosal surface is essential to host health, but excessive inflammation can lead to auto-inflammatory disorders like IBD. Our results suggest that the metabolite output of the microbiota, which is not only dependent on microbiota composition but also dietary intake, is a potential control point to drive long term adaptive immunity towards a homeostatic balance. Future research to determine the relationship between microbiota composition, dietary intake, metabolite prevalence, and immune status could provide powerful new therapeutic concepts for the modulation of the immune response. The ability to supplement

a patient's diet with safe, naturally occurring metabolites or metabolite-precursors could be a simple and elegant treatment approach to a number of inflammatory disorders that are currently treated with a generalized dampening of the global immune response. To get to this point, however, will require a non-trivial amount of data collection and bioinformatics concerning the diet, microbiota composition, metabolite output, and immune status.

CHAPTER IV

MODELING T CELL DIFFERENTIATION TO OPTIMIZE METABOLITE CONDITIONING IN VITRO*

Overview

Adoptive transfer of anti-inflammatory FOXP3+ Tregs either generated from a patient's naïve T cells or circulating FOXP3+ Tregs has gained attention as a new therapeutic strategy for auto-inflammatory disorders such as Inflammatory Bowel Disease. The isolated cells are conditioned *in vitro* to obtain a sufficient number of anti-inflammatory FOXP3+ Tregs that can be reintroduced into the patient and potentially reduce the pathologic inflammatory response. The previous results suggest that microbiota metabolites could be useful for conditioning cells during the *in vitro* expansion/differentiation step to be used for adoptive transfer therapy. However, determining a proper combination of cytokine and metabolite signals to obtain an optimal cell therapeutic introduces a large amount of unknown conditions that require a prohibitive amount of time to test empirically. To address this problem, we investigated the use of a neural network model to capture T cell dynamics and found:

1. A neural network model can be produced that models T cell culture outcome in response to both host cytokines and microbiota-metabolites.

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* Part of this chapter is reprinted with permission from Steinmeyer S, Howsmon DP, Alaniz RC, Hahn J, Jayaraman A. Empirical modeling of T cell activation predicts interplay of host cytokines and bacterial indole. Biotechnol Bioeng. 2017;114(11):2660-7. doi: 10.1002/bit.26371. Copyright 2017 John Wiley and Sons.

2. The generated neural network model predicts unexpected conditions for optimal Treg differentiation that are verifiable in our experimental system, supporting the utility of this model for investigation of metabolite and cytokine interactions.

Rationale

A neural network model is able to capture non-linear dynamics from simple input and output data. The basis of this model is the hidden layer, which is a series of nodes that take a given set of inputs (e.g. cytokine and metabolite concentrations) and based on optimized model parameters at each node, estimates the output (e.g. yield of FOXP3+ and IL-17+ T cells). When these nodes are combined, they can predict interactions between inputs that are not easily detected by simple examination of a set of experimental data and ultimately predict unexpected outcomes from culture conditions not initially tested. Importantly, this method of modeling does not require knowledge of mechanistic relationships between inputs, and that property makes this model very useful for our system of microbiota metabolite regulation of T cell differentiation, which is not completely characterized (135).

Results

Generating a neural network model to capture T cell differentiation dynamics

The first step in the process of building a neural network (NN) model is generating experimental training data, which should include the extremes of possible culture conditions (i.e. concentrations at minimum and maximum level of effect) and a limited amount of intermediate conditions. For example, we use one intermediate, one minimum, and one maximum concentration for each input in this model. We considered Treg- and Th17- skew cytokines (IL-

2, TGF-β, IL-6, and IL-23) as well as the metabolite particularly suited for generating an anti-inflammatory phenotype, indole. To avoid over-fitting of the model to our training data, which would decrease the ability of the model to predict untested culture condition outcomes, NNs with a single hidden layer and at most five neurons containing sigmoid transfer functions were evaluated.

We found that a neural network architecture with four hidden nodes captured our training data very well without the excessive error seen with the 3 node model or the potential to overfit our training data and not predict untested conditions very well seen in the 5 node model (fig. 37). Using our optimized 4 node NN model (fig. 38), we generated predictions for culture conditions that would be optimal for Treg generation. We determined a set of predicted conditions that would produce >50% FOXP3+ and <5% IL-17+ T cells at the end of a differentiation assay, which we will denote “optimal Treg yield” (fig. 39).

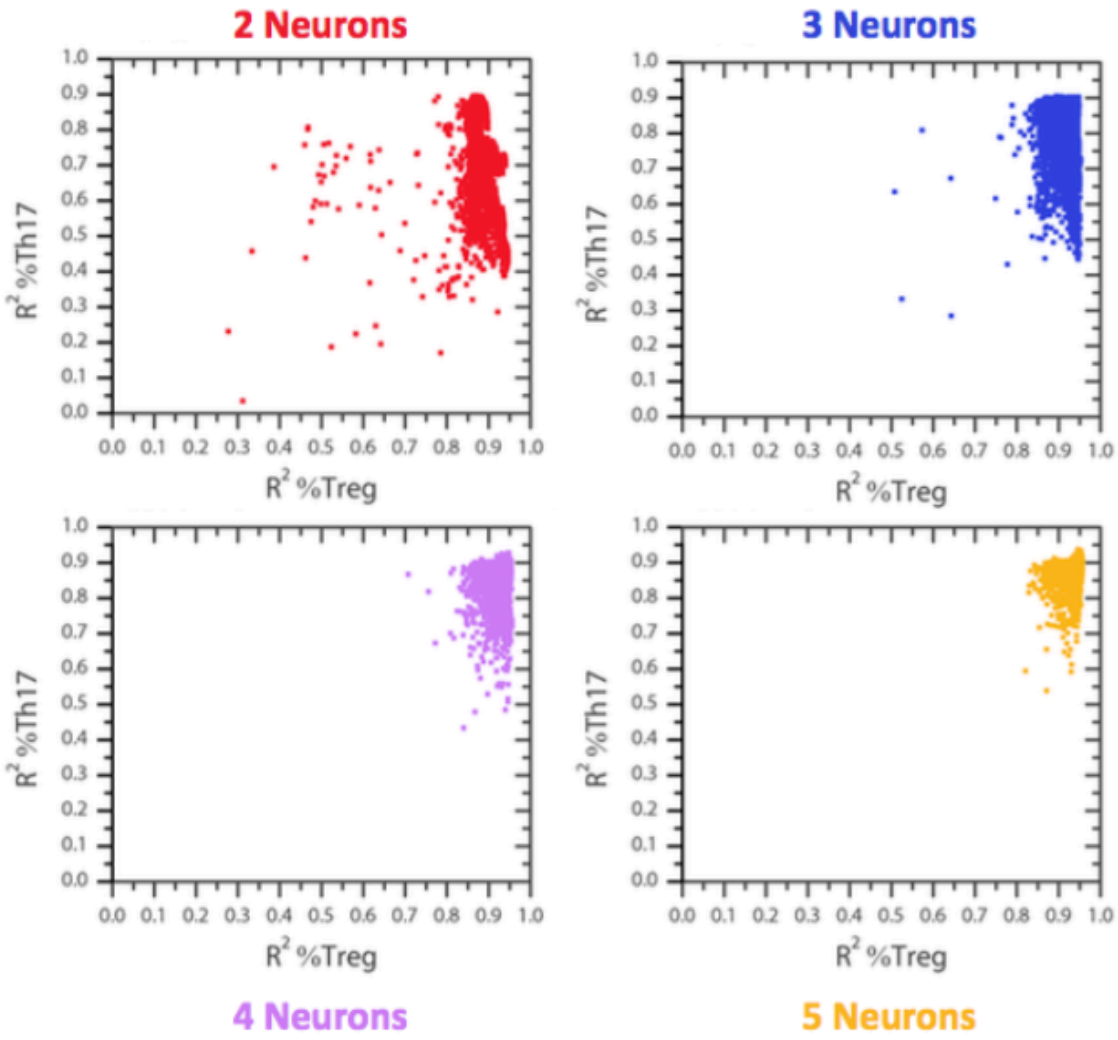

Figure 37. Analyzing different compositions of the hidden layer.

Neural networks were generated 10,000 times for each architecture (2, 3, 4, or 5 neurons in the hidden layer; input and output nodes stay the same). Each dot represents the R-squared value for prediction of Treg and Th17 differentiation in the testing data.

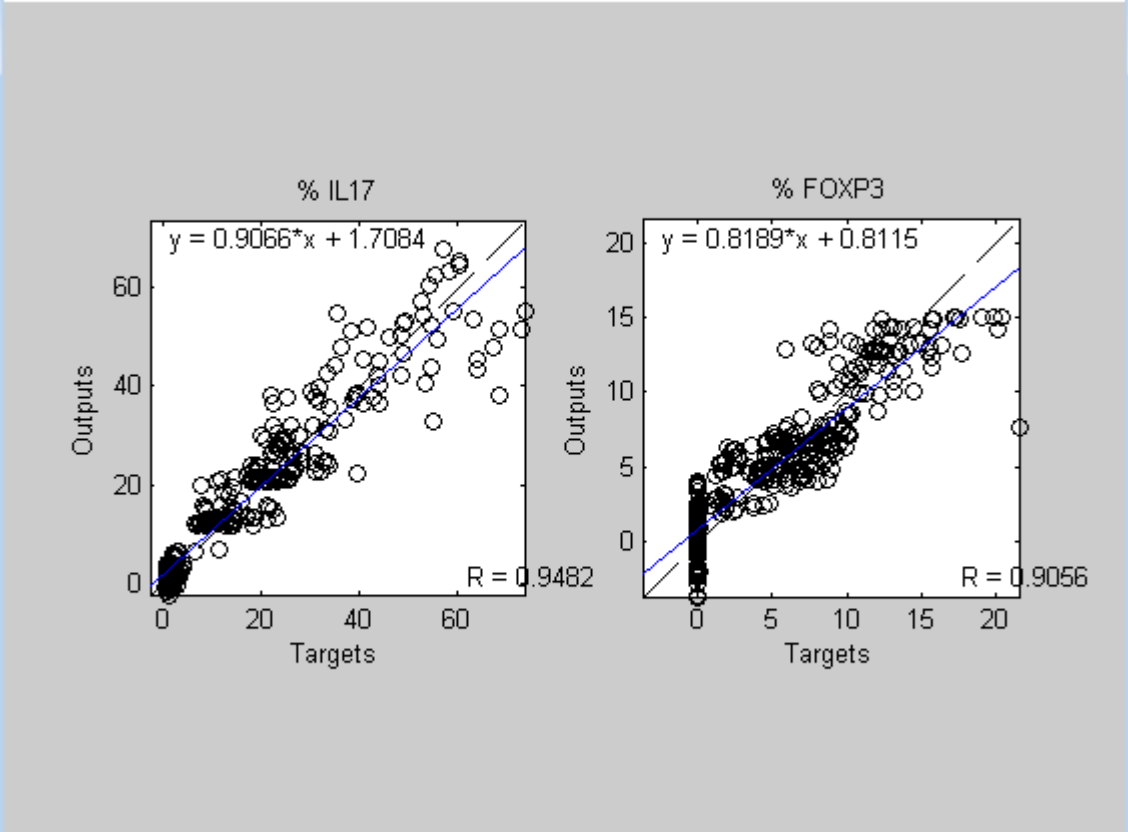

Figure 38. Testing a neural network model with optimized architecture.

The optimized neural network model predictions (outputs) were tested against experimental data (targets) for Treg and Th17 differentiation.

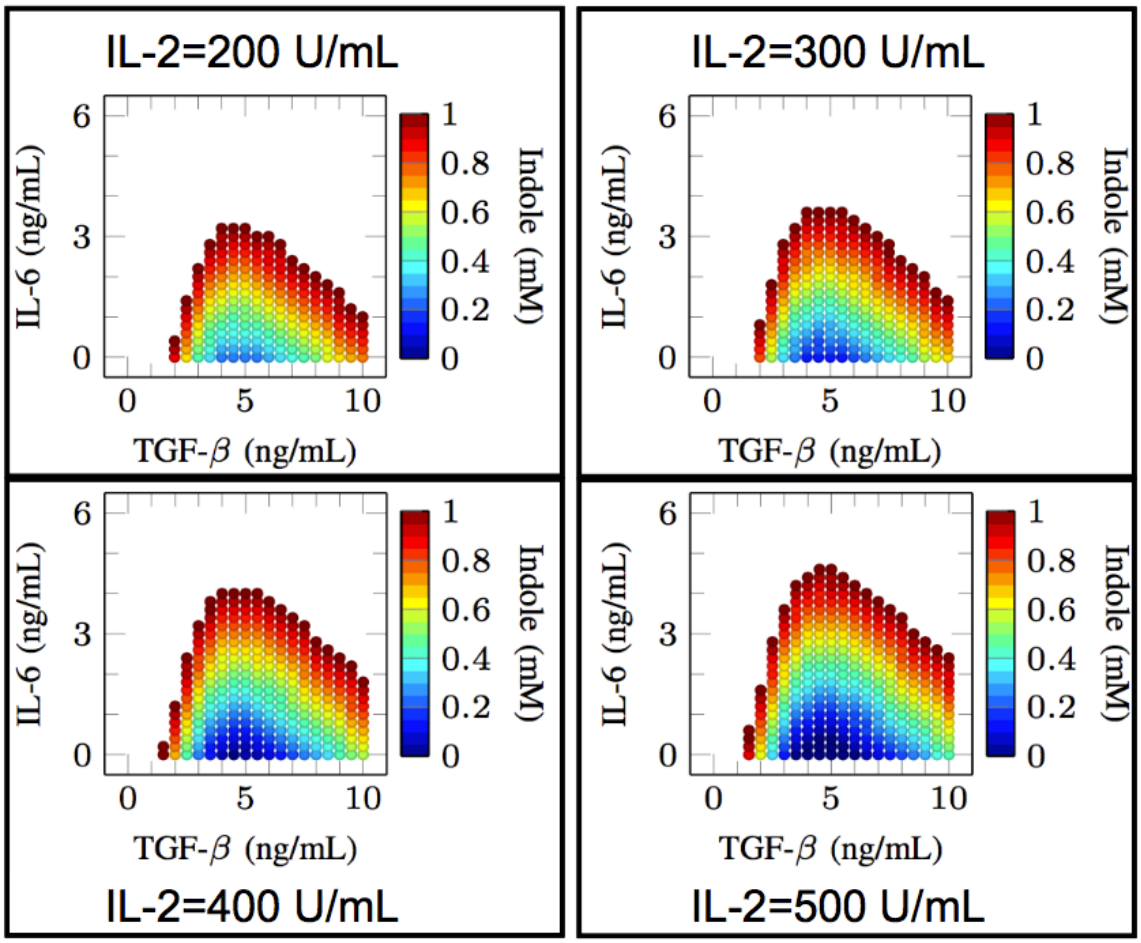

Figure 39. Neural network model predicts non-linear indole interaction with TGF-β, IL-6.

The 4 neuron neural network model was analyzed to predict culture conditions that promote a high yield of Tregs (>50%) and low yield of Th17 cells (<5%). Each dot is color coded for the minimum dose of indole required to achieve the desired culture outcome (high Tregs, low Th17 cells). The white space represents cytokine conditions that can't yield high Tregs and low Th17 for any dose of indole from 0 to 1 mM. Adapted from (135).

A few interesting trends emerge from the model. As expected, we see that IL-2 has a positive relationship with optimal Treg yield. As the concentration of IL-2 increases, the IL-6xTGF-β cytokine concentration space that results in optimal Treg yield increases in size. Furthermore, with increased IL-2, a lower amount of indole is required to obtain optimal Treg yield. This linear relationship is relatively intuitive and expected from the literature as well as our own experimental work.

A more unexpected result is the model's prediction of indole and TGF-β interaction. In the presence of a low to medium range concentration of inflammatory IL-6 (0-4 ng/mL), when optimal Treg yield is possible, TGF-β concentration is predicted to have a relationship with indole concentration such that as indole decreases from 1 mM, the range of TGF-β concentration required to obtain optimal Treg yield narrows towards 5 ng/mL. Note that a higher dose of indole (1 mM) can work with both a lower as well as a higher concentration of TGF-β.

We verified this model prediction by testing a higher resolution of indole concentrations with a low, intermediate, or high concentration of TGF-β (1 ng/mL, 5 ng/mL and 10 ng/mL). Indeed, we found that at the previously untested, intermediate concentration of 5 ng/mL, the lowest amount of indole optimally promotes Treg differentiation and inhibits Th17 differentiation, but at both a lower and higher TGF-β concentration, a higher concentration of indole was required to maximize Treg differentiation and minimize Th17 differentiation (fig. 40).

Interestingly, in the presence of low concentration pro-inflammatory IL-6 (3 ng/mL), the model predicted that a high indole concentration should still be able to maximize Treg and minimize

Th17 differentiation but only at an intermediate concentration of TGF-β. We verified this result as well, and we saw the same interaction between indole and TGF-β such that only at an intermediate TGF-β concentration can indole have maximal anti-inflammatory effects on T cell differentiation (fig. 41).

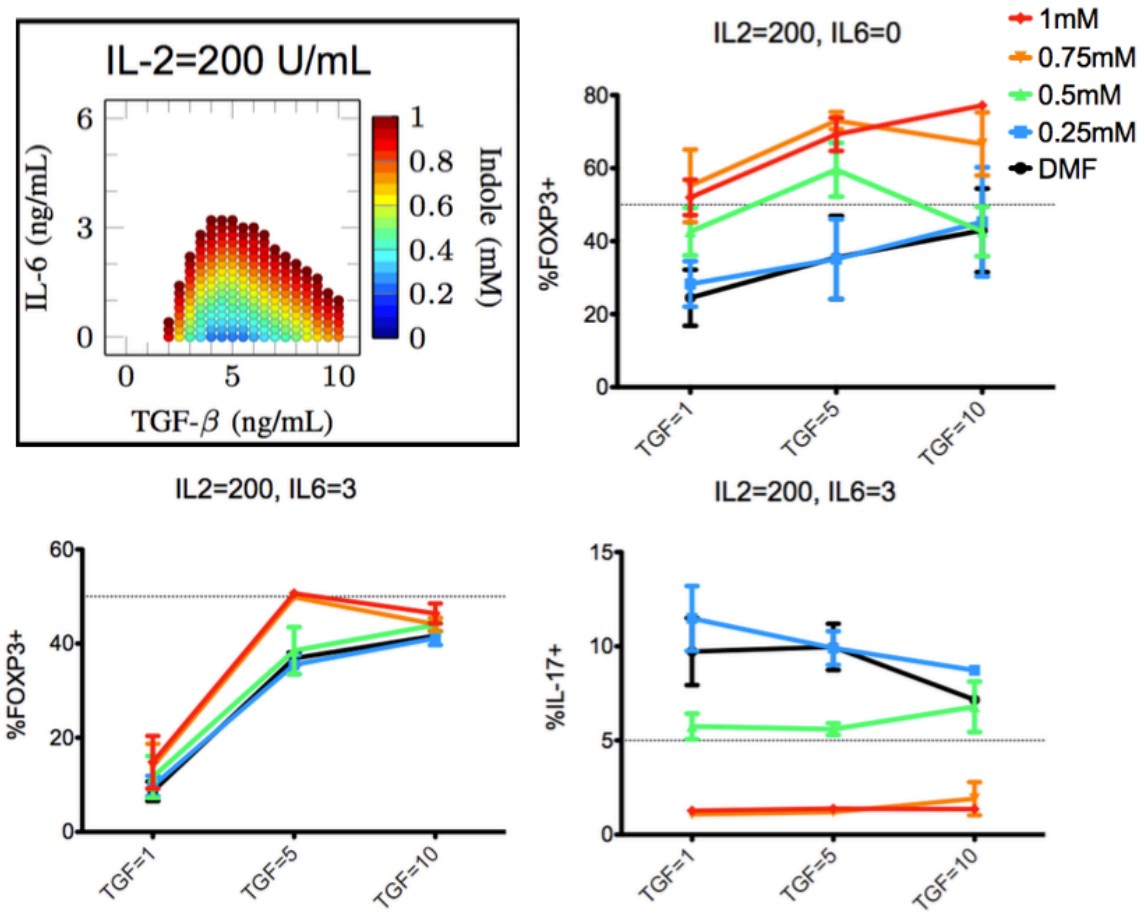

Figure 40. Neural network validation.

Adapted from (135).

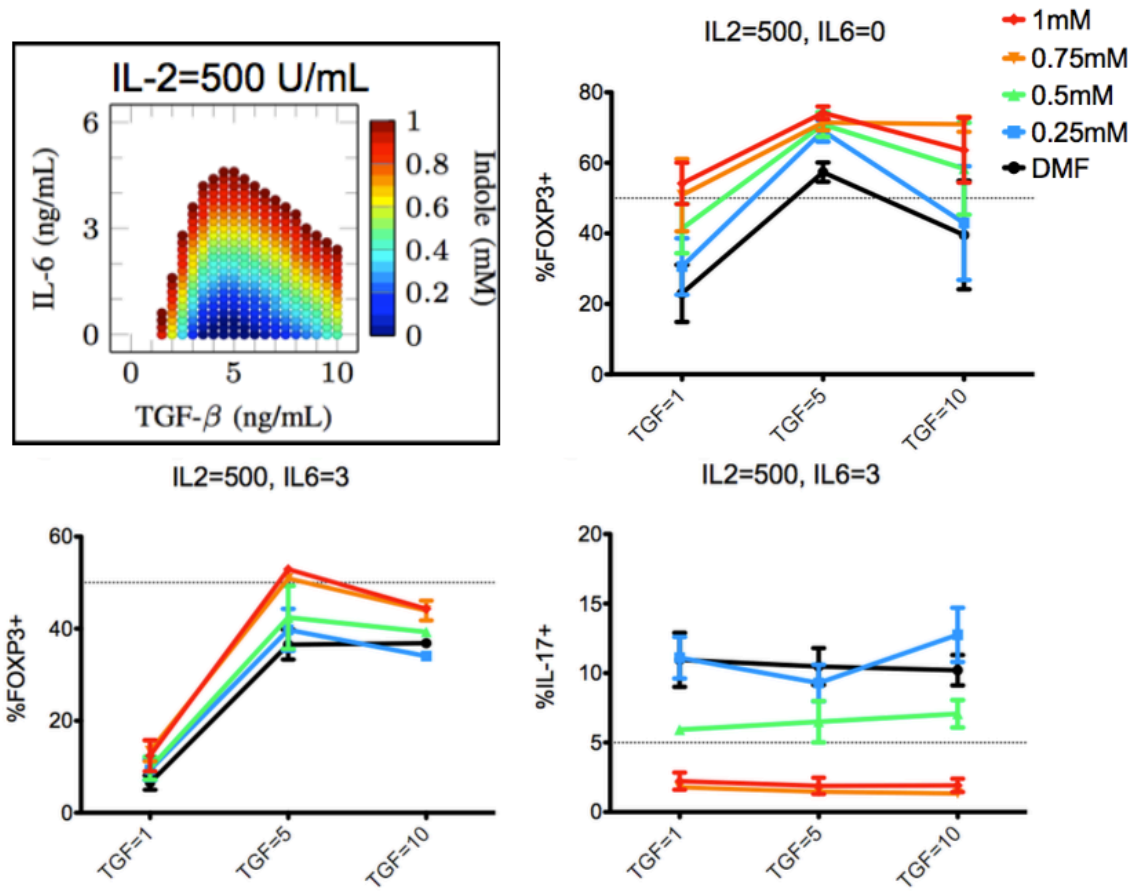

Figure 41. Neural network validation.

Adapted from (135).

Discussion

Using a neural network to build a "black-box" model of T cell differentiation proved computationally feasible and experimentally manageable. We cut the number of data points needed to empirically determine ideal Treg-skew conditions dramatically, and the neural network model we composed from the limited set of training data produced unexpected predictions about T cell activation in the presence of indole. This data supports the use of a neural network model for future analysis of microbiota metabolites found to modulate T cell differentiation or function. We expect that as the number of metabolites considered increases, as we addressed at the end of Chapter 1, this modeling approach will become extremely useful to predict how a repertoire of many metabolites interacts without having to empirically determine interaction. Even using 3 metabolites with different dose activity in four skew conditions, the number of experimental conditions reaches the capacity of a 96 well plate. By setting the foundation of neural network modeling as a viable approach to generate useful predictions about metabolite interactions, this work is a useful first step in our lab's study of tryptophan metabolites in T cell differentiation. The obvious next step of introducing more tryptophan metabolites will now be streamlined with our established protocol of modeling the interaction of culture conditions with a neural network.

CHAPTER V

CONCLUSION*

Microbiota-derived tryptophan metabolites have a powerful effect on T cell differentiation

We found in Chapter 1 that a number of tryptophan (Trp) metabolites produced form the microbiota are able to alter Treg and Th17 differentiation assay outcome. Indole and 5-hydroxyindole stood out as having the most robust anti-inflammatory effect (increased Treg, decreased Th17) and the only pro-inflammatory effect (increased Th17 and Th1, decreased Treg) of the tested Trp metabolites, respectively.

Our lab and others have identified the aryl hydrocarbon receptor (AhR) as a sensor of Trp metabolites produced in the GI tract, but our results suggest that indole and 5-hydroxyindole affect additional signaling pathways, including but not limited to mTOR. The long period of co-evolution vertebrates have shared with a microbiota makes it seem likely that we are only scratching the surface of host sensors for microbiota products, and a cell-wide transcriptomics approach could point to additional unexpected pathways that are modulated by the Trp metabolites.

Microbiota-derived short-chain fatty acids also impact T cell differentiation

Other groups have documented the effect of short-chain fatty acids (SCFAs) on T cell differentiation, and we found many similar results. What has not been touched on specifically is

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* Parts of this chapter are adapted with permission from Springer Nature: Current Allergy and Asthma Reports. Microbiota metabolite regulation of host immune homeostasis: a mechanistic missing link. Steinmeyer S, Lee K, Jayaraman A, Alaniz RC. Copyright 2015.

the ability of high dose butyrate to promote Th1 differentiation independently of host skew cytokines. This result stresses the conclusion that microbiota metabolites are a new class of "metabo-kine" with the ability to not only augment or inhibit ongoing host signaling pathways but to actually promote Th1 differentiation *de novo* just as IL-12 can.

Tryptophan metabolites and short-chain fatty acids can work together to promote tolerogenic T cell differentiation: butyrate dose is a deciding factor

The interaction of indole, 5-hydroxyindole, and butyrate generated an interesting picture of how a repertoire of metabolites in the GI tract might be able to promote oral tolerance during homeostasis. Specifically, we found that combining low dose butyrate with an active dose of 5-hydroxyindole and indole generated a largely anti-inflammatory effect on T cell differentiation: Th1 and Th17 differentiation were at baseline and Treg differentiation was augmented. This is particularly interesting when we consider that two of these signals (low dose butyrate and 5-hydroxyindole) promote Th1 and Th17 differentiation and two of these signals (low dose butyrate and indole) promote Treg differentiation. The non-linear interaction of these metabolites most likely provides only a glimpse of the complex interplay between microbiota metabolites during immune cell activation at the GI tract environmental interface. However, it does provide a strong basis to continue the study of microbiota metabolite signals in concert, rather than isolation.

Human T cells are affected by microbiota-derived metabolites during differentiation

An important caveat to the above results is that they are all generated in an inbred C57Bl/6 strain of mice. Not only are these not human, but they lack the genetic diversity found in a clinic's

patient population. Therefore, we took an important step in determining the clinical relevance of these metabolites by performing human T cell differentiation assays in the presence of microbiota-derived metabolites. We found that human Treg differentiation is affected by indole, 5-hydroxyindole and SCFAs in similar ways to our mouse cells. Human Th17 differentiation was promoted by 5-hydroxyindole, but due to a low baseline, we did not uncover any significant inhibition by indole. Admittedly, the sample size for this human study was very small, and with more time, we would like to gain a better understanding of how these metabolites interact with human T cells.

Regardless, these results support that the microbiota metabolism of dietary intake is a potential predictor of T cell differentiation status and subsequent inflammatory state, particularly in the gut-associated lymph tissue where these metabolites are predicted to be at the highest concentration. A prominent feature of microbiota research has been the diversity of microbiota composition in both healthy and sick subjects, but most attention has focused on the constituents of the microbiota rather than their functional role in metabolism. Further research is warranted to determine whether microbiota composition associated with excessive inflammation such as Inflammatory Bowel Disease (IBD) can be correlated to the population-level metabolism of an individual's microbiota. If the metabolic status of a patient's microbiota proves to be correlated to excessive inflammation, this work could contribute to a useful clinical marker and point of intervention. Additionally, this opens the door to consider the metabolites as potential conditioning agents for preparation of cellular therapeutics.

Microbiota-derived metabolites have acute and chronic effects on T cells

One important concern about cellular therapeutics is what happens to cells once they are reintroduced into the patient. With Treg adoptive transfer, there is a specific concern that once a Treg reaches the inflamed environment meant to be treated, the cell could be pushed toward a pro-inflammatory phenotype, ultimately exacerbating the inflammation it was meant to treat. We determined that not only do microbiota-derived metabolites affect acute activation of T cell *in vivo*—which supports our hypothesis that these signals modulate the GI tract mucosal immune system during homeostasis—but they also appear to affect the long term behavior of conditioned T cells and their progeny. Combined with our results in human T cell culture, this result strongly supports the investigation of microbiota-derived metabolites as a novel conditioning agent for cellular therapeutics. Much as antibiotic research has benefited greatly from natural products designed through evolutionary forces, the use of natural products from the microbiota is an untapped resource that could transform the emerging field of cellular therapy.

Neural network modeling is a novel approach to T cell culture optimization for downstream applications in cellular therapy

An inescapable quality of the gut microbiota is its extensive diversity and resultant complexity of metabolic function. The gut microbiota is arguably the true "organ" of first-pass metabolism that has profound effects on host health, and to mediate profound effects on a multicellular organism should require a diverse repertoire of metabolic products. Indeed, there is a large collection of metabolites not yet studied, produced by the gut microbiota, that may also modulate T cell function, and as the number of metabolites known to alter T cell function increases, the necessity of a computational tool to capture interaction between metabolites becomes hard to ignore.

We have made a first step in developing a modeling platform to capture the interaction between microbiota-derived metabolites and T cell differentiation that does not require prior knowledge of mechanistic targets, and this will allow rapid addition of new metabolites as they are discovered. By setting up a protocol to perform model development, we expect to incorporate additional Trp metabolites and SCFAs into the model with relative ease. The NN model is a major objective of our lab, as we have spent some time determining microbiota-derived Trp metabolites, and we are now focusing on cellular effects of the metabolites in concert. However, we believe this modeling approach will also be useful to the research community as new metabolite classes are identified that alter T cell function. By easing the workload of determining metabolite interaction, the model should be able to speed progress in understanding how the microbiota sends a net metabolic signal to the host to maintain tolerance or initiate an inflammatory response.

Closing remarks

The homeostasis achieved by the host and microbiota is an essential part of life. The co-evolution of these two entities has fostered a rich network of interaction between host cells and non-host cells, and one development of this process appears to be microbiota-derived metabolite regulation of T cell differentiation. The diverse array of enzymatic machinery possessed by a GI tract microbiota means that the repertoire of metabolites is vast, and much more work is needed to fully understand the metabolites produced by the microbiota and how they affect host health. Considerations such as relation to dietary intake, spatial location of microbiota constituents, and host sensitivity will all be crucial layers of understanding the role of microbiota metabolites in

holisitic organismal health. We have made a crucial first step with this work to describe two different classes of prominent metabolites, their singular and combined interactions, and the host cell machinery they act through to mediate their dramatic effects (fig. 42). Additionally, we have established a new modeling strategy to capture the unknown dynamics between microbiota metabolites, host cell cytokines, and T cell differentiation outcome, and this should facilitate future study of metabolite interaction as the pool of known immuno-active metabolites increases.

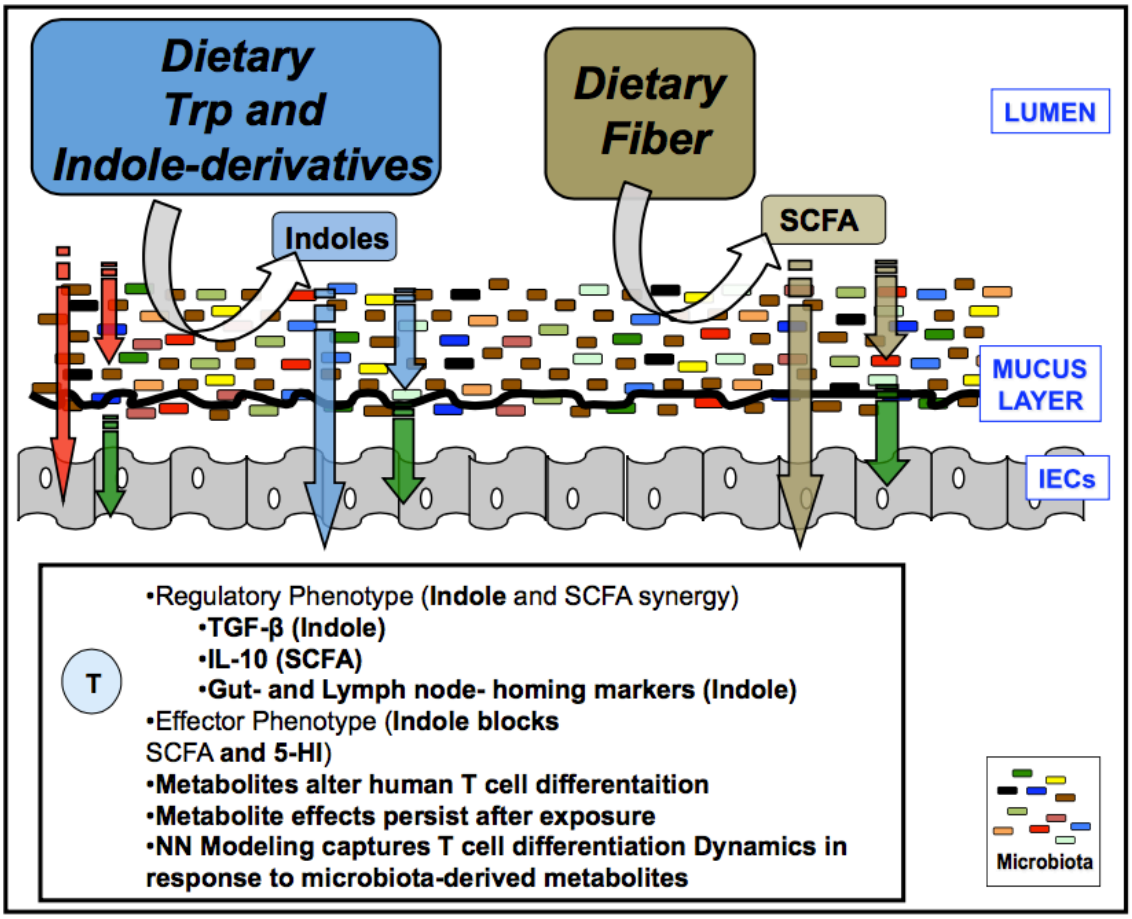

Figure 42. Global model of microbiota-derived metabolite modulation of T cell differentiation has expanded.

Contributions to model coming from this work in bold. Adapted from (1).

REFERENCES

1. Steinmeyer S, Lee K, Jayaraman A, Alaniz RC. Microbiota metabolite regulation of host immune homeostasis: a mechanistic missing link. Curr Allergy Asthma Rep. 2015;15(5):24. doi: 10.1007/s11882-015-0524-2.

2. Mowat AM, Agace WW. Regional specialization within the intestinal immune system. Nature reviews Immunology. 2014;14(10):667-85. doi: 10.1038/nri3738.

3. Weiner HL, da Cunha AP, Quintana F, Wu H. Oral tolerance. Immunol Rev. 2011;241(1):241-59. doi: 10.1111/j.1600-065X.2011.01017.x.

4. Ashida H, Ogawa M, Kim M, Mimuro H, Sasakawa C. Bacteria and host interactions in the gut epithelial barrier. Nat Chem Biol. 2011;8(1):36-45. doi: 10.1038/nchembio.741.

5. Spees AM, Lopez CA, Kingsbury DD, Winter SE, Baumler AJ. Colonization resistance: battle of the bugs or Menage a Trois with the host? PLoS pathogens. 2013;9(11):e1003730. doi: 10.1371/journal.ppat.1003730.

6. Wells JM, Rossi O, Meijerink M, van Baarlen P. Epithelial crosstalk at the microbiota-mucosal interface. Proceedings of the National Academy of Sciences of the United States of America. 2011;108 Suppl 1:4607-14. doi: 10.1073/pnas.1000092107.

7. Howitt MR, Lavoie S, Michaud M, Blum AM, Tran SV, Weinstock JV, Gallini CA, Redding K, Margolskee RF, Osborne LC, Artis D, Garrett WS. Tuft cells, taste-chemosensory cells, orchestrate parasite type 2 immunity in the gut. Science. 2016;351(6279):1329-33. doi: 10.1126/science.aaf1648.

8. Grainger JR, Askenase MH, Guimont-Desrochers F, da Fonseca DM, Belkaid Y. Contextual functions of antigen-presenting cells in the gastrointestinal tract. Immunol Rev. 2014;259(1):75-87. doi: 10.1111/imr.12167.

9. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, Koyasu S, Locksley RM, McKenzie AN, Mebius RE, Powrie F, Vivier E. Innate lymphoid cells--a proposal for uniform nomenclature. Nature reviews Immunology. 2013;13(2):145-9. doi: 10.1038/nri3365.

10. Tait Wojno ED, Artis D. Innate lymphoid cells: balancing immunity, inflammation, and tissue repair in the intestine. Cell host & microbe. 2012;12(4):445-57. doi: 10.1016/j.chom.2012.10.003.

11. Gasteiger G, Rudensky AY. Interactions between innate and adaptive lymphocytes. Nature reviews Immunology. 2014;14(9):631-9. doi: 10.1038/nri3726.

12. Bilate AM, Lafaille JJ. Induced CD4+Foxp3+ regulatory T cells in immune tolerance. Annual review of immunology. 2012;30:733-58. doi: 10.1146/annurev-immunol-020711-075043.

13. Weaver CT, Elson CO, Fouser LA, Kolls JK. The Th17 pathway and inflammatory diseases of the intestines, lungs, and skin. Annual review of pathology. 2013;8:477-512. doi: 10.1146/annurev-pathol-011110-130318.

14. Kaplan MH. Th9 cells: differentiation and disease. Immunol Rev. 2013;252(1):104-15. doi: 10.1111/imr.12028.

15. Kim KS, Hong SW, Han D, Yi J, Jung J, Yang BG, Lee JY, Lee M, Surh CD. Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. Science. 2016;351(6275):858-63. doi: 10.1126/science.aac5560.

16. Jiang S, Dong C. A complex issue on CD4(+) T-cell subsets. Immunol Rev. 2013;252(1):5-11. doi: 10.1111/imr.12041.

17. Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio CW, Santacruz N, Peterson DA, Stappenbeck TS, Hsieh CS. Peripheral education of the immune system by colonic commensal microbiota. Nature. 2011;478(7368):250-4. doi: 10.1038/nature10434.

18. Cebula A, Seweryn M, Rempala GA, Pabla SS, McIndoe RA, Denning TL, Bry L, Kraj P, Kisielow P, Ignatowicz L. Thymus-derived regulatory T cells contribute to tolerance to commensal microbiota. Nature. 2013;497(7448):258-62. doi: 10.1038/nature12079.

19. Rescigno M, Lopatin U, Chieppa M. Interactions among dendritic cells, macrophages, and epithelial cells in the gut: implications for immune tolerance. Current opinion in immunology. 2008;20(6):669-75. doi: 10.1016/j.coi.2008.09.007.

20. Carvalho FA, Aitken JD, Vijay-Kumar M, Gewirtz AT. Toll-like receptor-gut microbiota interactions: perturb at your own risk! Annual review of physiology. 2012;74:177-98. doi: 10.1146/annurev-physiol-020911-153330.

21. Chassaing B, Darfeuille-Michaud A. The commensal microbiota and enteropathogens in the pathogenesis of inflammatory bowel diseases. Gastroenterology. 2011;140(6):1720-28. doi: 10.1053/j.gastro.2011.01.054.

22. Cho JH. The genetics and immunopathogenesis of inflammatory bowel disease. Nature reviews Immunology. 2008;8(6):458-66. doi: 10.1038/nri2340.

23. Kaur N, Chen CC, Luther J, Kao JY. Intestinal dysbiosis in inflammatory bowel disease. Gut microbes. 2011;2(4):211-6. doi: 10.4161/gmic.2.4.17863.

24. Tamboli CP, Neut C, Desreumaux P, Colombel JF. Dysbiosis in inflammatory bowel disease. Gut. 2004;53(1):1-4.

25. Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. Annual review of immunology. 2010;28:573-621. doi: 10.1146/annurev-immunol-030409-101225.

26. Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature. 2011;474(7351):298-306. doi: 10.1038/nature10208.

27. Man SM, Kaakoush NO, Mitchell HM. The role of bacteria and pattern-recognition receptors in Crohn's disease. Nature reviews Gastroenterology & hepatology. 2011;8(3):152-68. doi: 10.1038/nrgastro.2011.3.

28. Baumgart DC, Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. Lancet. 2007;369(9573):1641-57. doi: 10.1016/S0140-6736(07)60751-X.

29. Fischbach MA, Bluestone JA, Lim WA. Cell-based therapeutics: the next pillar of medicine. Science translational medicine. 2013;5(179):179ps7. doi: 10.1126/scitranslmed.3005568.

30. Himmel ME, Yao Y, Orban PC, Steiner TS, Levings MK. Regulatory T-cell therapy for inflammatory bowel disease: more questions than answers. Immunology. 2012;136(2):115-22. doi: 10.1111/j.1365-2567.2012.03572.x.

31. Fantini MC, Becker C, Tubbe I, Nikolaev A, Lehr HA, Galle P, Neurath MF. Transforming growth factor beta induced FoxP3+ regulatory T cells suppress Th1 mediated experimental colitis. Gut. 2006;55(5):671-80. doi: 10.1136/gut.2005.072801.

32. Haribhai D, Lin W, Edwards B, Ziegelbauer J, Salzman NH, Carlson MR, Li SH, Simpson PM, Chatila TA, Williams CB. A central role for induced regulatory T cells in tolerance induction in experimental colitis. J Immunol. 2009;182(6):3461-8. doi: 10.4049/jimmunol.0802535.

33. Hippen KL, Merkel SC, Schirm DK, Nelson C, Tennis NC, Riley JL, June CH, Miller JS, Wagner JE, Blazar BR. Generation and large-scale expansion of human inducible regulatory T cells that suppress graft-versus-host disease. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2011;11(6):1148-57. doi: 10.1111/j.1600-6143.2011.03558.x.

34. Hippen KL, Riley JL, June CH, Blazar BR. Clinical perspectives for regulatory T cells in transplantation tolerance. Seminars in immunology. 2011;23(6):462-8. doi: 10.1016/j.smim.2011.07.008.

35. Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. Nature reviews Immunology. 2007;7(8):585-98. doi: 10.1038/nri2138.

36. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, Defor T, Levine BL, June CH, Rubinstein P, McGlave PB, Blazar BR, Wagner JE. Infusion of ex vivo

expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. Blood. 2011;117(3):1061-70. doi: 10.1182/blood-2010-07-293795.

37. Bluestone JA, Buckner JH, Fitch M, Gitelman SE, Gupta S, Hellerstein MK, Herold KC, Lares A, Lee MR, Li K, Liu W, Long SA, Masiello LM, Nguyen V, Putnam AL, Rieck M, Sayre PH, Tang Q. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. Science translational medicine. 2015;7(315).

38. Eastaff-Leung N, Mabarrack N, Barbour A, Cummins A, Barry S. Foxp3+ regulatory T cells, Th17 effector cells, and cytokine environment in inflammatory bowel disease. Journal of clinical immunology. 2010;30(1):80-9. doi: 10.1007/s10875-009-9345-1.

39. Kalden JR, Burkhardt H. Autoimmune Disease: Treatment. Encyclopedia of Life Sciences: John Wiley & Sons, Ltd; 2009.

40. Schmitt EG, Haribhai D, Williams JB, Aggarwal P, Jia S, Charbonnier LM, Yan K, Lorier R, Turner A, Ziegelbauer J, Georgiev P, Simpson P, Salzman NH, Hessner MJ, Broeckel U, Chatila TA, Williams CB. IL-10 produced by induced regulatory T cells (iTregs) controls colitis and pathogenic ex-iTregs during immunotherapy. J Immunol. 2012;189(12):5638-48. doi: 10.4049/jimmunol.1200936.

41. Yamane H, Paul WE. Early signaling events that underlie fate decisions of naive CD4+ T cells toward distinct T-helper subsets. Immunol Rev. 2013;252:12-23.

42. Hong T, Xing J, Li L, Tyson JJ. A mathematical model for the reciprocal differentiation of T helper 17 cells and induced regulatory T cells. PLoS computational biology. 2011;7(7):e1002122. doi: 10.1371/journal.pcbi.1002122.

43. Yang XO, Nurieva R, Martinez GJ, Kang HS, Chung Y, Pappu BP, Shah B, Chang SH, Schluns KS, Watowich SS, Feng XH, Jetten AM, Dong C. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. Immunity. 2008;29(1):44-56. doi: 10.1016/j.immuni.2008.05.007.

44. Zhou L, Lopes JE, Chong MM, Ivanov, II, Min R, Victora GD, Shen Y, Du J, Rubtsov YP, Rudensky AY, Ziegler SF, Littman DR. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. Nature. 2008;453(7192):236-40. doi: 10.1038/nature06878.

45. Zheng SG. Regulatory T cells vs Th17: differentiation of Th17 versus Treg, are they mutually exclusive? Am J Clin Exp Immunol. 2013;2(1):94-106.

46. Barbi J, Pardoll D, Pan F. Metabolic control of the Treg/Th17 axis. Immunol Rev. 2013;252(1):52-77. doi: 10.1111/imr.12029.

47. Gerriets VA, Rathmell JC. Metabolic pathways in T cell fate and function. Trends in immunology. 2012;33(4):168-73. doi: 10.1016/j.it.2012.01.010.

48. Yurchenko E, Shio MT, Huang TC, Da Silva Martins M, Szyf M, Levings MK, Olivier M, Piccirillo CA. Inflammation-driven reprogramming of CD4+ Foxp3+ regulatory T cells into pathogenic Th1/Th17 T effectors is abrogated by mTOR inhibition in vivo. PLoS One. 2012;7(4):e35572. doi: 10.1371/journal.pone.0035572.

49. Frank DN, Zhu W, Sartor RB, Li E. Investigating the biological and clinical significance of human dysbioses. Trends in microbiology. 2011;19(9):427-34. doi: 10.1016/j.tim.2011.06.005.

50. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J, Xu J, Li S, Li D, Cao J, Wang B, Liang H, Zheng H, Xie Y, Tap J, Lepage P, Bertalan M, Batto JM, Hansen T, Le Paslier D, Linneberg A, Nielsen HB, Pelletier E, Renault P, Sicheritz-Ponten T, Turner K, Zhu H, Yu C, Li S, Jian M, Zhou Y, Li Y, Zhang X, Li S, Qin N, Yang H, Wang J, Brunak S, Dore J, Guarner F, Kristiansen K, Pedersen O, Parkhill J, Weissenbach J, Meta HITC, Bork P, Ehrlich SD, Wang J. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59-65. doi: 10.1038/nature08821.

51. Ding T, Schloss PD. Dynamics and associations of microbial community types across the human body. Nature. 2014;509(7500):357-60. doi: 10.1038/nature13178.

52. Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, Almeida M, Arumugam M, Batto JM, Kennedy S, Leonard P, Li J, Burgdorf K, Grarup N, Jorgensen T, Brandslund I, Nielsen HB, Juncker AS, Bertalan M, Levenez F, Pons N, Rasmussen S, Sunagawa S, Tap J, Tims S, Zoetendal EG, Brunak S, Clement K, Dore J, Kleerebezem M, Kristiansen K, Renault P, Sicheritz-Ponten T, de Vos WM, Zucker JD, Raes J, Hansen T, Meta HITc, Bork P, Wang J, Ehrlich SD, Pedersen O. Richness of human gut microbiome correlates with metabolic markers. Nature. 2013;500(7464):541-6. doi: 10.1038/nature12506.

53. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Science translational medicine. 2009;1(6):6ra14. doi: 10.1126/scitranslmed.3000322.

54. Cotillard A, Kennedy SP, Kong LC, Prifti E, Pons N, Le Chatelier E, Almeida M, Quinquis B, Levenez F, Galleron N, Gougis S, Rizkalla S, Batto JM, Renault P, consortium ANRM, Dore J, Zucker JD, Clement K, Ehrlich SD. Dietary intervention impact on gut microbial gene richness. Nature. 2013;500(7464):585-8. doi: 10.1038/nature12480.

55. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, Siuzdak G. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(10):3698-703. doi: 10.1073/pnas.0812874106.

56. Sharon G, Garg N, Debelius J, Knight R, Dorrestein PC, Mazmanian SK. Specialized metabolites from the microbiome in health and disease. Cell metabolism. 2014;20(5):719-30. doi: 10.1016/j.cmet.2014.10.016.

57. Greenblum S, Turnbaugh PJ, Borenstein E. Metagenomic systems biology of the human gut microbiome reveals topological shifts associated with obesity and inflammatory bowel disease. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(2):594-9. doi: 10.1073/pnas.1116053109.

58. Sridharan GV, Choi K, Klemashevich C, Wu C, Prabakaran D, Pan LB, Steinmeyer S, Mueller C, Yousofshahi M, Alaniz RC, Lee K, Jayaraman A. Prediction and quantification of bioactive microbiota metabolites in the mouse gut. Nature communications. 2014;5:5492. doi: 10.1038/ncomms6492.

59. Vinolo MA, Rodrigues HG, Nachbar RT, Curi R. Regulation of inflammation by short chain fatty acids. Nutrients. 2011;3(10):858-76. doi: 10.3390/nu3100858.

60. Donohoe DR, Garge N, Zhang X, Sun W, O'Connell TM, Bunger MK, Bultman SJ. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell metabolism. 2011;13(5):517-26. doi: 10.1016/j.cmet.2011.02.018.

61. Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, Muir AI, Wigglesworth MJ, Kinghorn I, Fraser NJ, Pike NB, Strum JC, Steplewski KM, Murdock PR, Holder JC, Marshall FH, Szekeres PG, Wilson S, Ignar DM, Foord SM, Wise A, Dowell SJ. The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. The Journal of biological chemistry. 2003;278(13):11312-9. doi: 10.1074/jbc.M211609200.

62. Davie JR. Inhibition of histone deacetylase activity by butyrate. The Journal of nutrition. 2003;133(7 Suppl):2485S-93S.

63. Waldecker M, Kautenburger T, Daumann H, Busch C, Schrenk D. Inhibition of histone-deacetylase activity by short-chain fatty acids and some polyphenol metabolites formed in the colon. The Journal of nutritional biochemistry. 2008;19(9):587-93. doi: 10.1016/j.jnutbio.2007.08.002.

64. Cousens LS, Gallwitz D, Alberts BM. Different accessibilities in chromatin to histone acetylase. The Journal of biological chemistry. 1979;254(5):1716-23.

65. Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, Brezillon S, Dupriez V, Vassart G, Van Damme J, Parmentier M, Detheux M. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. The Journal of biological chemistry. 2003;278(28):25481-9. doi: 10.1074/jbc.M301403200.

66. Pluznick JL, Protzko RJ, Gevorgyan H, Peterlin Z, Sipos A, Han J, Brunet I, Wan LX, Rey F, Wang T, Firestein SJ, Yanagisawa M, Gordon JI, Eichmann A, Peti-Peterdi J, Caplan MJ. Olfactory receptor responding to gut microbiota-derived signals plays a role in renin secretion and blood pressure regulation. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(11):4410-5. doi: 10.1073/pnas.1215927110.

67. Kimura I, Inoue D, Maeda T, Hara T, Ichimura A, Miyauchi S, Kobayashi M, Hirasawa A, Tsujimoto G. Short-chain fatty acids and ketones directly regulate sympathetic nervous

system via G protein-coupled receptor 41 (GPR41). Proceedings of the National Academy of Sciences of the United States of America. 2011;108(19):8030-5. doi: 10.1073/pnas.1016088108.

68. Soret R, Chevalier J, De Coppet P, Poupeau G, Derkinderen P, Segain JP, Neunlist M. Short-chain fatty acids regulate the enteric neurons and control gastrointestinal motility in rats. Gastroenterology. 2010;138(5):1772-82. doi: 10.1053/j.gastro.2010.01.053.

69. Nankova BB, Agarwal R, MacFabe DF, La Gamma EF. Enteric bacterial metabolites propionic and butyric acid modulate gene expression, including CREB-dependent catecholaminergic neurotransmission, in PC12 cells--possible relevance to autism spectrum disorders. PLoS One. 2014;9(8):e103740. doi: 10.1371/journal.pone.0103740.

70. Fung KY, Cosgrove L, Lockett T, Head R, Topping DL. A review of the potential mechanisms for the lowering of colorectal oncogenesis by butyrate. The British journal of nutrition. 2012;108(5):820-31. doi: 10.1017/S0007114512001948.

71. Masui R, Sasaki M, Funaki Y, Ogasawara N, Mizuno M, Iida A, Izawa S, Kondo Y, Ito Y, Tamura Y, Yanamoto K, Noda H, Tanabe A, Okaniwa N, Yamaguchi Y, Iwamoto T, Kasugai K. G protein-coupled receptor 43 moderates gut inflammation through cytokine regulation from mononuclear cells. Inflammatory bowel diseases. 2013;19(13):2848-56. doi: 10.1097/01.MIB.0000435444.14860.ea.

72. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, Schilter HC, Rolph MS, Mackay F, Artis D, Xavier RJ, Teixeira MM, Mackay CR. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature. 2009;461(7268):1282-6. doi: 10.1038/nature08530.

73. Chang PV, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(6):2247-52. doi: 10.1073/pnas.1322269111.

74. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, Blanchard C, Junt T, Nicod LP, Harris NL, Marsland BJ. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nature medicine. 2014;20(2):159-66. doi: 10.1038/nm.3444.

75. Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, Shi H, Thangaraju M, Prasad PD, Manicassamy S, Munn DH, Lee JR, Offermanns S, Ganapathy V. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. Immunity. 2014;40(1):128-39. doi: 10.1016/j.immuni.2013.12.007.

76. Kim MH, Kang SG, Park JH, Yanagisawa M, Kim CH. Short-chain fatty acids activate GPR41 and GPR43 on intestinal epithelial cells to promote inflammatory responses in mice. Gastroenterology. 2013;145(2):396-406 e1-10. doi: 10.1053/j.gastro.2013.04.056.

77. Gaudier E, Jarry A, Blottiere HM, de Coppet P, Buisine MP, Aubert JP, Laboisse C, Cherbut C, Hoebler C. Butyrate specifically modulates MUC gene expressio in intestinal

eptihelial goblet cells deprived of glucose. American journal of physiology Gastrointestinal and liver physiology. 2004;287:G1168-G74. doi: 10.1152/ajpgi.00219.2004.-The.

78. Ohata A, Usami M, Miyoshi M. Short-chain fatty acids alter tight junction permeability in intestinal monolayer cells via lipoxygenase activation. Nutrition. 2005;21(7-8):838-47. doi: 10.1016/j.nut.2004.12.004.

79. Vinolo MA, Rodrigues HG, Hatanaka E, Sato FT, Sampaio SC, Curi R. Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. The Journal of nutritional biochemistry. 2011;22(9):849-55. doi: 10.1016/j.jnutbio.2010.07.009.

80. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, Glickman JN, Garrett WS. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science. 2013;341(6145):569-73. doi: 10.1126/science.1241165.

81. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke JM, Topping DL, Tomita M, Hori S, Ohara O, Morita T, Koseki H, Kikuchi J, Honda K, Hase K, Ohno H. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 2013;504(7480):446-50. doi: 10.1038/nature12721.

82. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, Rudensky AY. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature. 2013;504(7480):451-5. doi: 10.1038/nature12726.

83. Park J, Kim M, Kang SG, Jannasch AH, Cooper B, Patterson J, Kim CH. Short-chain fatty acids induce both effector and regulatory T cells by suppression of histone deacetylases and regulation of the mTOR-S6K pathway. Mucosal immunology. 2015;8(1):80-93. doi: 10.1038/mi.2014.44.

84. Chi H. Regulation and function of mTOR signalling in T cell fate decisions. Nature reviews Immunology. 2012;12(5):325-38. doi: 10.1038/nri3198.

85. Nguyen NT, Nakahama T, Le DH, Van Son L, Chu HH, Kishimoto T. Aryl hydrocarbon receptor and kynurenine: recent advances in autoimmune disease research. Frontiers in immunology. 2014;5:551. doi: 10.3389/fimmu.2014.00551.

86. Hisamatsu T, Okamoto S, Hashimoto M, Muramatsu T, Andou A, Uo M, Kitazume MT, Matsuoka K, Yajima T, Inoue N, Kanai T, Ogata H, Iwao Y, Yamakado M, Sakai R, Ono N, Ando T, Suzuki M, Hibi T. Novel, objective, multivariate biomarkers composed of plasma amino acid profiles for the diagnosis and assessment of inflammatory bowel disease. PLoS One. 2012;7(1):e31131. doi: 10.1371/journal.pone.0031131.

87. Schicho R, Nazyrova A, Shaykhutdinov R, Duggan G, Vogel HJ, Storr M. Quantitative metabolomic profiling of serum and urine in DSS-induced ulcerative colitis of mice by (1)H NMR spectroscopy. J Proteome Res. 2010;9(12):6265-73. doi: 10.1021/pr100547y.

88. Jin UH, Lee SO, Sridharan G, Lee K, Davidson LA, Jayaraman A, Chapkin RS, Alaniz R, Safe S. Microbiome-derived tryptophan metabolites and their aryl hydrocarbon receptor-dependent agonist and antagonist activities. Molecular pharmacology. 2014;85(5):777-88. doi: 10.1124/mol.113.091165.

89. Zelante T, Iannitti RG, Cunha C, De Luca A, Giovannini G, Pieraccini G, Zecchi R, D'Angelo C, Massi-Benedetti C, Fallarino F, Carvalho A, Puccetti P, Romani L. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. Immunity. 2013;39(2):372-85. doi: 10.1016/j.immuni.2013.08.003.

90. Basu R, O'Quinn DB, Silberger DJ, Schoeb TR, Fouser L, Ouyang W, Hatton RD, Weaver CT. Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria. Immunity. 2012;37(6):1061-75. doi: 10.1016/j.immuni.2012.08.024.

91. Denison MS, Nagy SR. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol. 2003;43:309-34.

92. Gu YZ, Hogenesch JB, Bradfield CA. The PAS superfamily: sensors of environmental and developmental signals. Annu Rev Pharmacol Toxicol. 2000;40:519-61.

93. Quintana FJ, Basso AS, Iglesias AH, Korn T, Farez MF, Bettelli E, Caccamo M, Oukka M, Weiner HL. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. Nature. 2008;453(7191):65-71. doi: 10.1038/nature06880.

94. Lee YK, Mukasa R, Hatton RD, Weaver CT. Developmental plasticity of Th17 and Treg cells. Current opinion in immunology. 2009;21(3):274-80. doi: 10.1016/j.coi.2009.05.021.

95. Stockinger B, Di Meglio P, Gialitakis M, Duarte JH. The aryl hydrocarbon receptor: multitasking in the immune system. Annual review of immunology. 2014;32:403-32. doi: 10.1146/annurev-immunol-032713-120245.

96. Li Y, Innocentin S, Withers DR, Roberts NA, Gallagher AR, Grigorieva EF, Wilhelm C, Veldhoen M. Exogenous stimuli maintain intraepithelial lymphocytes via aryl hydrocarbon receptor activation. Cell. 2011;147(3):629-40. doi: 10.1016/j.cell.2011.09.025.

97. Kiss EA, Vonarbourg C, Kopfmann S, Hobeika E, Finke D, Esser C, Diefenbach A. Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. Science. 2011;334(6062):1561-5. doi: 10.1126/science.1214914.

98. Hepworth MR, Monticelli LA, Fung TC, Ziegler CG, Grunberg S, Sinha R, Mantegazza AR, Ma HL, Crawford A, Angelosanto JM, Wherry EJ, Koni PA, Bushman FD, Elson CO, Eberl G, Artis D, Sonnenberg GF. Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. Nature. 2013;498(7452):113-7. doi: 10.1038/nature12240.

99. Hahn ME, Karchner SI, Evans BR, Franks DG, Merson RR, Lapseritis JM. Unexpected diversity of aryl hydrocarbon receptors in non-mammalian vertebrates: insights from comparative genomics. J Exp Zool A Comp Exp Biol. 2006;305(9):693-706. doi: 10.1002/jez.a.323.

100. Li G, Young KD. Indole production by the tryptophanase TnaA in Escherichia coli is determined by the amount of exogenous tryptophan. Microbiology. 2013;159(Pt 2):402-10. doi: 10.1099/mic.0.064139-0.

101. Lee JH, Lee J. Indole as an intercellular signal in microbial communities. FEMS microbiology reviews. 2010;34(4):426-44. doi: 10.1111/j.1574-6976.2009.00204.x.

102. Bansal T, Alaniz RC, Wood TK, Jayaraman A. The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(1):228-33. doi: 10.1073/pnas.0906112107.

103. Shimada Y, Kinoshita M, Harada K, Mizutani M, Masahata K, Kayama H, Takeda K. Commensal bacteria-dependent indole production enhances epithelial barrier function in the colon. PLoS One. 2013;8(11):e80604. doi: 10.1371/journal.pone.0080604.

104. Zuccato E, Venturi M, Di Leo G, Colombo L, Bertolo C, Doldi SB, Mussini E. Role of bile acids and metabolic activity of colonic bacteria in increased risk of colon cancer after cholecystectomy. Dig Dis Sci. 1993;38(3):514-9.

105. Karlin DA, Mastromarino AJ, Jones RD, Stroehlein JR, Lorentz O. Fecal skatole and indole and breath methane and hydrogen in patients with large bowel polyps or cancer. J Cancer Res Clin Oncol. 1985;109(2):135-41.

106. Yanofsky C, Horn V, Gollnick P. Physiological Studies of Tryptphan Transport and Tryptophanase Operon Induction in Escherichia coli. Journal of bacteriology. 1991;173(19):6009-17.

107. Bansal T, Englert D, Lee J, Hegde M, Wood TK, Jayaraman A. Differential effects of epinephrine, norepinephrine, and indole on Escherichia coli O157:H7 chemotaxis, colonization, and gene expression. Infection and immunity. 2007;75(9):4597-607. doi: 10.1128/IAI.00630-07.

108. Venkatesh M, Mukherjee S, Wang H, Li H, Sun K, Benechet AP, Qiu Z, Maher L, Redinbo MR, Phillips RS, Fleet JC, Kortagere S, Mukherjee P, Fasano A, Le Ven J, Nicholson JK, Dumas ME, Khanna KM, Mani S. Symbiotic bacterial metabolites regulate gastrointestinal barrier function via the xenobiotic sensor PXR and Toll-like receptor 4. Immunity. 2014;41(2):296-310. doi: 10.1016/j.immuni.2014.06.014.

109. Vodovotz Y, Constantine G, Rubin J, Csete M, Voit EO, An G. Mechanistic simulations of inflammation: current state and future prospects. Mathematical biosciences. 2009;217(1):1-10. doi: 10.1016/j.mbs.2008.07.013.

110. Germain RN, Meier-Schellersheim M, Nita-Lazar A, Fraser ID. Systems biology in immunology: a computational modeling perspective. Annual review of immunology. 2011;29:527-85. doi: 10.1146/annurev-immunol-030409-101317.

111. MacKay DJC. Bayesian Interpolation. Neural Comput. 1992;4:415-47.

112. MacKay DJC. A Practical Bayesian Framework for Backpropagation Networks. Neural Comput. 1992;4:448-72.

113. Frankel DS, Frankel SL, Binder BJ, Vogt RF. Application of neural networks to flow cytometry data analysis and real-time cell classification. Cytometry. 1996;23(4):290-302. doi: 10.1002/(SICI)1097-0320(19960401)23:4<290::AID-CYTO5>3.0.CO;2-L.

114. Nielsen M, Lundegaard C, Worning P, Lauemoller SL, Lamberth K, Buus S, Brunak S, Lund O. Reliable prediction of T-cell epitopes using neural networks with novel sequence representations. Protein science : a publication of the Protein Society. 2003;12(5):1007-17. doi: 10.1110/ps.0239403.

115. Qian N, Sejnowski TJ. Predicting the Secondary Structure of Globular Proteins Using Neural Network Models. J Mol Biol. 1988;202:865-84.

116. Ye K, Jin S, Shimizu K. Fuzzy Neural Network for the Control of High Cell Density Cultivation of Recombinant Escherichia coli. J Ferment Bioeng. 1994;77(6):663-73.

117. Fontenelle B, Gilbert KM. n-Butyrate anergized effector CD4+ T cells independent of regulatory T cell generation or activity. Scandinavian journal of immunology. 2012;76(5):457-63. doi: 10.1111/j.1365-3083.2012.02740.x.

118. Butz NV, Gronostajski RM, Campbell CE. T-box proteins differentially activate the expression of the endogenous interferon gamma gene versus transfected reporter genes in non-immune cells. Gene. 2006;377:130-9. doi: 10.1016/j.gene.2006.03.016.

119. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. Immunity. 2004;21(4):527-38. doi: 10.1016/j.immuni.2004.08.011.

120. Hill JA, Hall JA, Sun CM, Cai Q, Ghyselinck N, Chambon P, Belkaid Y, Mathis D, Benoist C. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi Cells. Immunity. 2008;29(5):758-70. doi: 10.1016/j.immuni.2008.09.018.

121. Ma J, Liu Y, Li Y, Gu J, Liu J, Tang J, Wang J, Ryffel B, Shen Y, Brand D, Liu Z, Zheng SG. Differential role of all-trans retinoic acid in promoting the development of CD4+ and CD8+ regulatory T cells. Journal of leukocyte biology. 2014;95(2):275-83. doi: 10.1189/jlb.0513297.

122. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nature reviews Immunology. 2008;8(7):523-32. doi: 10.1038/nri2343.

123. Campbell DJ, Koch MA. Phenotypical and functional specialization of FOXP3+ regulatory T cells. Nature reviews Immunology. 2011;11(2):119-30. doi: 10.1038/nri2916.

124. Rubtsov YP, Niec RE, Josefowicz S, Li L, Darce J, Mathis D, Benoist C, Rudensky AY. Stability of the regulatory T cell lineage in vivo. Science. 2010;329(5999):1667-71. doi: 10.1126/science.1191996.

125. Dang EV, Barbi J, Yang HY, Jinasena D, Yu H, Zheng Y, Bordman Z, Fu J, Kim Y, Yen HR, Luo W, Zeller K, Shimoda L, Topalian SL, Semenza GL, Dang CV, Pardoll DM, Pan F. Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1. Cell. 2011;146(5):772-84. doi: 10.1016/j.cell.2011.07.033.

126. Clambey ET, McNamee EN, Westrich JA, Glover LE, Campbell EL, Jedlicka P, de Zoeten EF, Cambier JC, Stenmark KR, Colgan SP, Eltzschig HK. Hypoxia-inducible factor-1 alpha-dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(41):E2784-93. doi: 10.1073/pnas.1202366109.

127. Shehade H, Acolty V, Moser M, Oldenhove G. Cutting Edge: Hypoxia-Inducible Factor 1 Negatively Regulates Th1 Function. J Immunol. 2015;195(4):1372-6. doi: 10.4049/jimmunol.1402552.

128. de Wit J, Souwer Y, van Beelen AJ, de Groot R, Muller FJ, Klaasse Bos H, Jorritsma T, Kapsenberg ML, de Jong EC, van Ham SM. CD5 costimulation induces stable Th17 development by promoting IL-23R expression and sustained STAT3 activation. Blood. 2011;118(23):6107-14. doi: 10.1182/blood-2011-05-352682.

129. Ayyoub M, Raffin C, Valmori D. Generation of Th17 from human naive CD4+ T cells preferentially occurs from FOXP3+ Tregs upon costimulation via CD28 or CD5. Blood. 2012;119(20). doi: 10.1182/blood-2012-02-409722.

130. Farez MF, Mascanfroni ID, Mendez-Huergo SP, Yeste A, Murugaiyan G, Garo LP, Balbuena Aguirre ME, Patel B, Ysrraelit MC, Zhu C, Kuchroo VK, Rabinovich GA, Quintana FJ, Correale J. Melatonin Contributes to the Seasonality of Multiple Sclerosis Relapses. Cell. 2015;162(6):1338-52. doi: 10.1016/j.cell.2015.08.025.

131. Wu C, Yosef N, Thalhamer T, Zhu C, Xiao S, Kishi Y, Regev A, Kuchroo VK. Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. Nature. 2013;496(7446):513-7. doi: 10.1038/nature11984.

132. Zhou L, Chong MM, Littman DR. Plasticity of CD4+ T cell lineage differentiation. Immunity. 2009;30(5):646-55. doi: 10.1016/j.immuni.2009.05.001.

133. Evans CM, Jenner RG. Transcription factor interplay in T helper cell differentiation. Briefings in functional genomics. 2013;12(6):499-511. doi: 10.1093/bfgp/elt025.

134. Scotta C, Esposito M, Fazekasova H, Fanelli G, Edozie FC, Ali N, Xiao F, Peakman M, Afzali B, Sagoo P, Lechler RI, Lombardi G. Differential effects of rapamycin and retinoic acid on expansion, stability and suppressive qualities of human CD4(+)CD25(+)FOXP3(+) T regulatory cell subpopulations. Haematologica. 2013;98(8):1291-9. doi: 10.3324/haematol.2012.074088.

135. Steinmeyer S, Howsmon DP, Alaniz RC, Hahn J, Jayaraman A. Empirical modeling of T cell activation predicts interplay of host cytokines and bacterial indole. Biotechnol Bioeng. 2017;114(11):2660-7. doi: 10.1002/bit.26371.

APPENDIX

METHODS AND SUPPLEMENTAL FIGURES

Methods

Cell isolation

CD4+ CD25- or CD4+ CD25- CD44lo CD62Lhi T cells were isolated to high purity (>98%) from the pooled spleen and mesenteric lymph nodes of wild-type, $AhR^{-/-}$, or FOXP3-GFP C57BL/6 mice (Jackson Labs and Taconic Labs) with a BD FACS Aria II flow cytometer. Fc Block (BD), αCD4-efluor450 (eBioscience clone GK1.5), αCD25-PECy7 (eBioscience clone PC61.5), αCD44-APC (eBioscience clone IM7), and αCD62L-FITC (eBioscience clone MEL-14) antibodies were used for staining before sorting.

For Isolation of FOXP3-GFP+ iTregs after culture, cells were washed and resuspended in PBS + 0.5% bovine serum albumin, and GFP+ cells were isolated to high purity (>98%) with a BD FACS Aria II.

Human CD4+ CD45RO- untouched naïve T cells were isolated using the RoboSep human naïve T cell isolation kin on a Robosep automated device (StemCell Cat. # 19155F).

In vitro T cell differentiation

Isolated mouse cells were cultured at an initial concentration of $1x10^5$ cells/well in RPMI 1640 supplemented with 2-mercaptoethanol, gentamicin, penicillin, streptomycin, and 10% FBS (all from Life Technologies) in a 96-well round bottom plate (Falcon) coated with 5 μg/mL αCD3

(BioXcell clone 145-2C11) and 2 μg/mL αCD28 (BioXcell clone 37.51) for 72 hours. For Th1-skew: 5 ng/mL IL-12 (Peprotech cat. #210-12) and 10 μg/mL αIL-4 (BioXCell clone 11B11) were added to culture. For Th2-skew: 10 ng/mL IL-4 (Peprotech) and 10 μg/mL αIFN-γ (BioXCell clone R4-6A2) were added to culture. For Th17-skew: 10 ng/mL IL-6 (Peprotech), 5 ng/mL IL-23 (R&D), 0.5 ng/mL TGF-β (Peprotech), 10 μg/mL αIL-4 (BioXCell clone 11B11), and 10 μg/mL αIFN-γ (BioXCell clone R4-6A2) were added to culture. For Treg-skew: 2 ng/mL TGF-β (Peprotech) and 100 U/mL IL-2 (Roche) were added to culture.

Isolated human cells were cultured at an initial concentration of $1x10^5$ cells/well in RPMI 1640 supplemented with 2-mercaptoethanol, gentamicin, penicillin, streptomycin, and 10% FBS (all from Life Technologies) in a 96-well flat bottom plate (Falcon) coated with 10 μg/mL αCD3 (BD Biosciences clone UCHT1) and 2 μg/mL αCD28 (BD Biosciences clone CD28.2). for 7-10 days until wells reached confluency. For Th17-skew: 20 ng/mL IL-1β (BD Biosciences), 30 ng/mL IL-6 (Peprotech), 30 ng/mL IL-23 (Peprotech) and 0.1 ng/mL TGF-β (Peprotech) were added to culture. For Treg-skew: 1 ng/mL TGF-β (Peprotech) and 10 U/mL IL-2 (Roche) were added to culture.

Sodium propionate, sodium acetate, sodium butyrate and sodium chloride (all from Sigma-Aldrich) were solubilized in media and added to culture. Indole and 5-hydroxyindole were solubilized in DMF (both from Sigma-Aldrich) at 2M and 0.05M, respectively, and brought to final concentration in media. MGCD0103 (Selleck Chemicals) was solubilized in DMF at 2 mM and brought to final concentration in media. A 0.05% DMF solvent control was used for all experiments.

Intracellular cytokine and transcription factor staining

For the last four hours of culture, cells were treated with golgi plug (BD), PMA and ionomycin (both from Sigma-Aldrich). Cells were fixed with the FOXP3 fixation buffer (eBioscience) or 4% paraformaldehyde (Sigma-Aldrich), treated with permeabilization buffer (eBioscience) and stained with appropriate combinations of the following antibodies: αIL-17a-PE or –APC (eBioscience clone eBio17B7 or BD Biosciences clone N49-653), αFOXP3-APC or –FITC (eBioscience clone FJK-16s or BD Biosciences clone 259D/C7), αIL-4-FITC (eBioscience clone BVD6-24G2)**,** and αIFN-γ-PE or –APC (eBioscience clone XMG1.2).

iTreg suppression assay and cytokine expression analysis

Purified FOXP3-GFP+ T cells from indole-, butyate, or DMF solvent control- conditioned Treg induction cultures were used for supernatant production and suppression assays. For supernatant production, cells were plated at $1x10^6$ cells/mL in a 24 well plate coated with αCD3 in cell culture media supplemented with IL-2. After 18 hours, supernatant was collected for ELISA analysis of IL-10 and TGF-β secretion. For suppression assays, iTregs were plated at 1:1, 1:4, 1:16, 0:1, and 1:0 (iTreg:effector CD8+ T cell) in a 48 well plate coated with αCD3. After 72 hours, supernatant was collected and analyzed for IL-2 concentration by ELISA.

Acute T cell activation in vivo

15 μg of αCD3 (BioXcell clone 145-2C11) was administered via intra-peritoneal injection at 0 and 48 hours. Along with αCD3; butyrate, indole, a combination of butyrate and indole, or a 17% DMSO (all from Sigma-Aldrich) vehicle control were co-administered in the same injection at 0 and 48 hours. At 52 hours, spleen and mesenteric lymph nodes were isolated and plated

individually at a concentration of $1x10^6$ cells/well with RPMI media supplemented with 2-mercaptoethanol, gentamicin, penicillin, and streptomycin + 10% FCS (all from Life Technologies) in a 96-well round bottom plate (Falcon). Cells were immediately stimulated for fours hours with PMA and ionomycin in the presence of golgi plug followed by intracellular staining.

Th17 transfer colitis

$RAG1^{-/-}$ mice on a C57Bl/6 background (Jackson Labs) received by intraperitoneal injection $1x10^6$ unpurified cells from a Th17 induction culture treated with DMF solvent control, 25 μM 5-hydroxyindole, or 1 mM indole. Cells were washed and resuspended in warm PBS(+)(+) prior to injection. Weight was recorded weekly until weight loss began, at which point weight was recorded every other day. At day 15 and day 49 Mesenteric lymph nodes were isolated and plated individually at a concentration of $1x10^6$ cells/well with RPMI media supplemented with 2-mercaptoethanol, gentamicin, penicillin, and streptomycin + 10% FBS (all from Life Technologies) in a 96-well round bottom plate (Falcon). Cells were immediately stimulated for fours hours with PMA and ionomycin in the presence of golgi plug followed by intracellular staining.

Neural network modeling

The Matlab NNToolbox was used to create and analyze Neural Network structures. The inputs were considered to be concentrations of IL-2, IL-6, IL-23, TGF-β, and indole, and the outputs were the percentage of IL-17+ and FOXP3+ cells determined by flow cytometry after our typical 72 hour differentiation assay. The data was partitioned into extreme points and intermediate

points. To avoid extrapolation, all of the extreme points were kept in the training set. The interior points were then partitioned randomly such that 70%, 15%, and 15% of the total data were used for training, validation, and testing, respectively. Of 10,000 such partitions, representative Neural Networks with good performance and regression statistics (low error in both validation and testing) were isolated for further analysis.

Supplemental figures

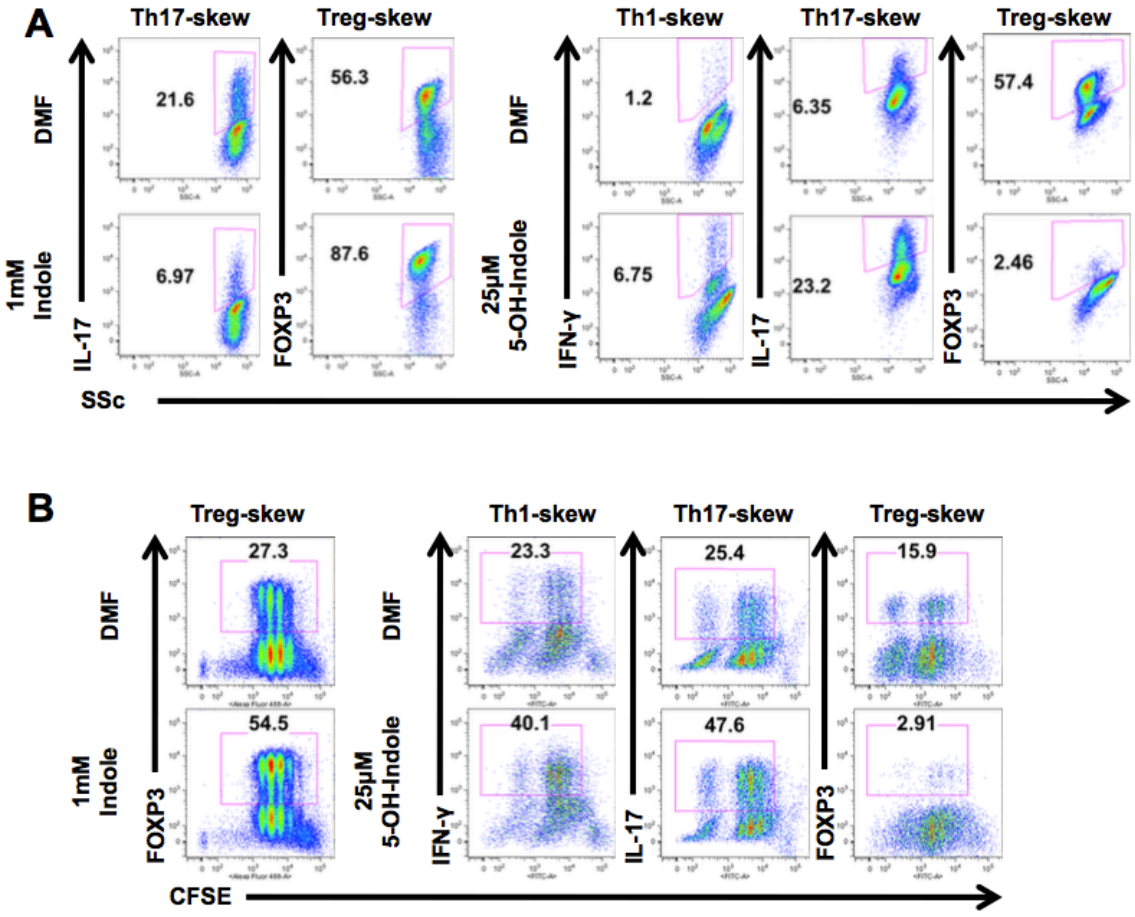

Figure 43. Trp metabolites modulate CD44lo CD62Lhi T cell differentiation and induce proliferation regulatory or effector T cells.

CD44lo CD62lHI CD4+ T cells sorted to high purity (A) or CD4+ CD25- T cells sorted to high purity and stained with CFSE (B) were cultured with TCR-crosslinking for 72 hours in the presence of Th1-, Th17-, or Treg- skew cytokines and indicated concentration indole, 5-hydroxyIndole, or DMF solvent control. At the end of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug followed by intracellular staining.

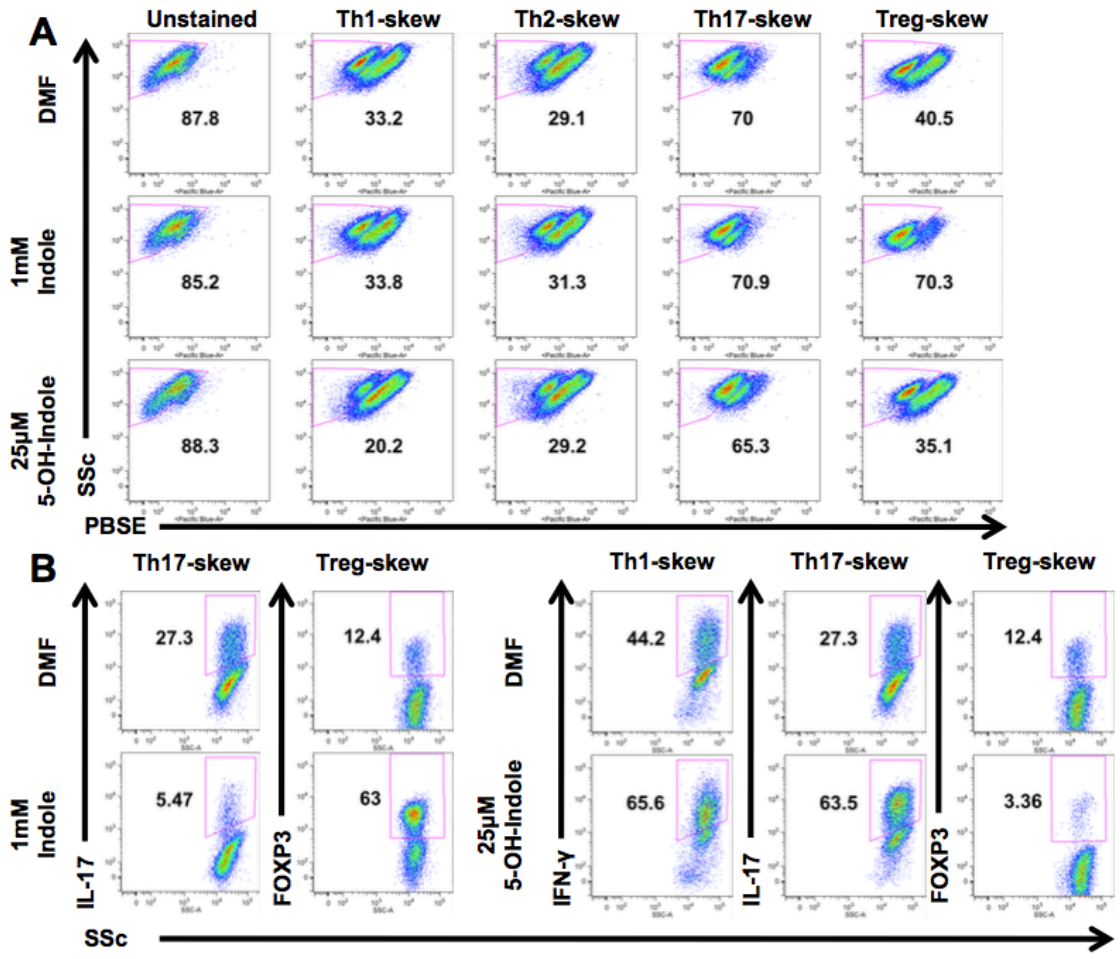

Figure 44. Trp metabolites do not cause toxic effects during T cell differentiation.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th1-, Th2-, Th17-, or Treg- skew cytokines and indicated concentration indole, 5-hydroxyIndole, or DMF solvent control. At the end of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug followed by intracellular staining for intracellular and PBSE Live/dead discrimination dye. (A) shows PBSE staining, (B) shows cytokine and transcription factor staining in PBSE negative population.

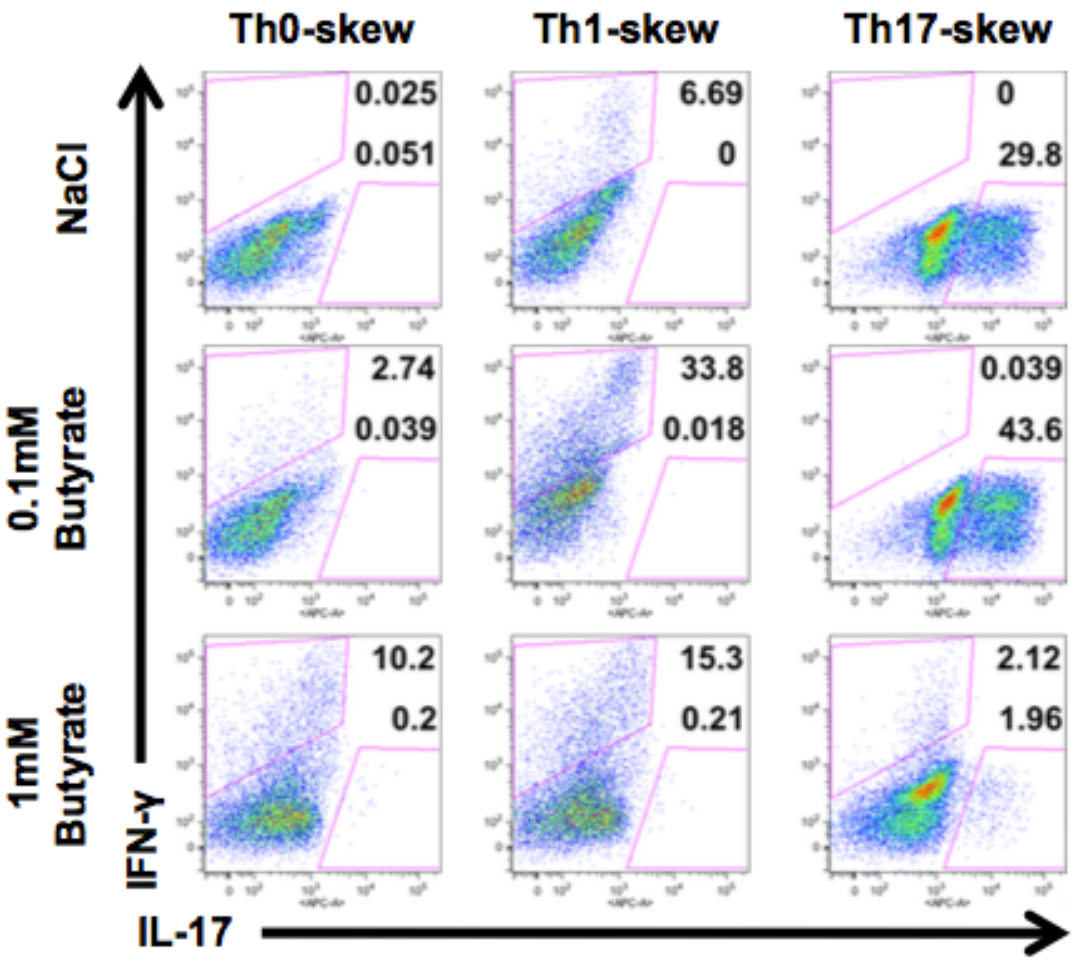

Figure 45. Butyrate modulates CD44lo CD62Lhi T cell differentiation.

CD44lo CD62lHI CD4+ T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th1- or Th17- skew cytokines and indicated concentration butyrate or NaCl control. At the end of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug followed by intracellular staining.

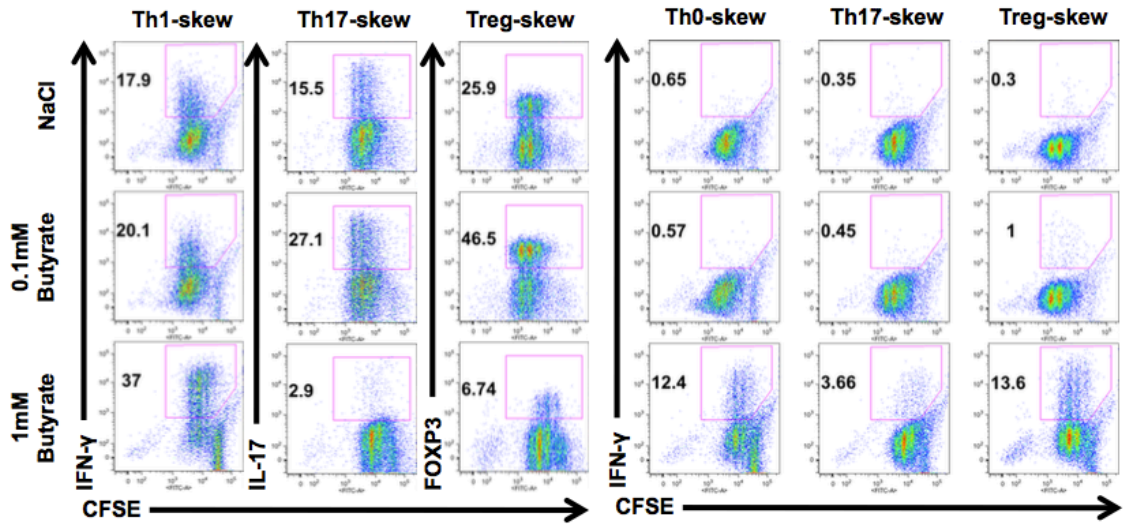

Figure 46. Butyrate induces proliferating regulatory and effector T cells.

CD4+ CD25- T cells sorted to high purity and stained with CFSE were cultured with TCR-crosslinking for 72 hours in the presence of Th1-, Th17-, or Treg- skew cytokines and indicated concentration butyrate or NaCl control. At the end of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug followed by intracellular staining.

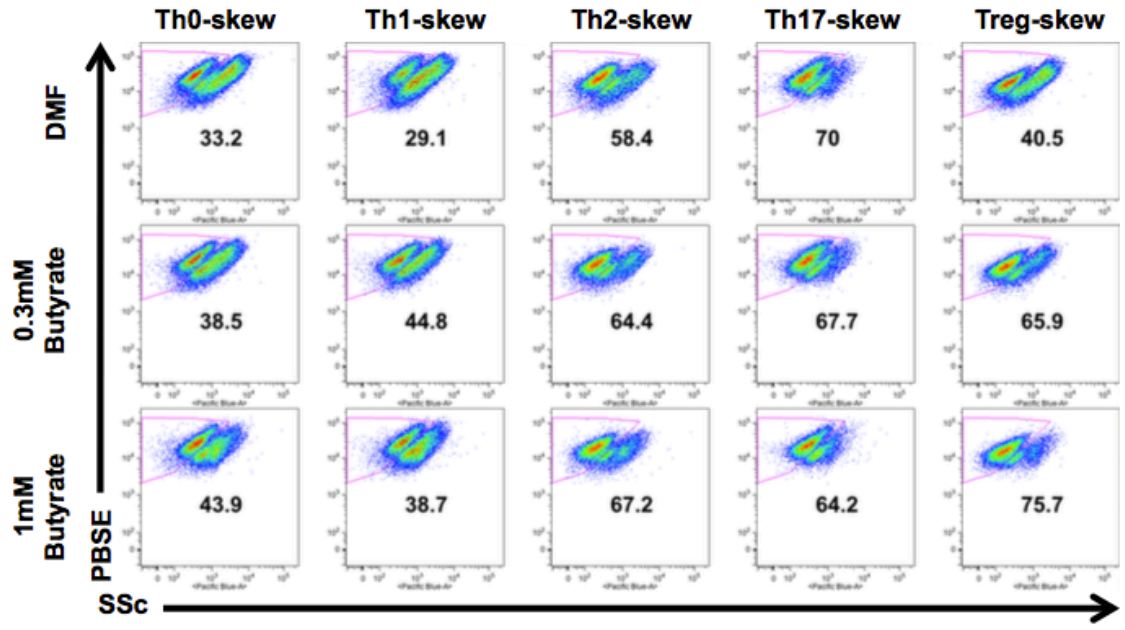

Figure 47. Butyrate does not cause toxic effects during T cell differentiation.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th1-, Th2-, Th17-, or Treg- skew cytokines and indicated concentration butyrate or NaCl control. At the end of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug followed by intracellular staining for intracellular and PBSE Live/dead discrimination dye.

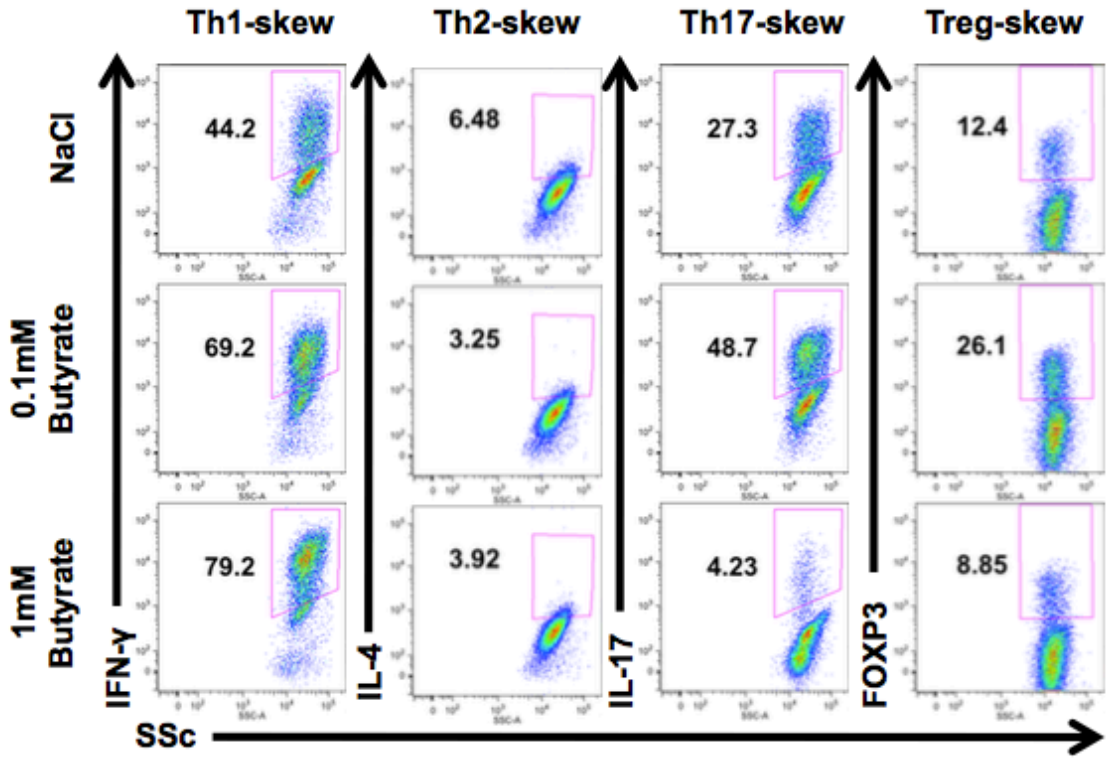

Figure 48. Butyrate does not cause toxic effects during T cell differentiation.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th1-, Th2-, Th17-, or Treg- skew cytokines and indicated concentration butyrate or NaCl control. At the end of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug followed by intracellular staining for intracellular and PBSE Live/dead discrimination dye. Data shown is from PBSE negative population.

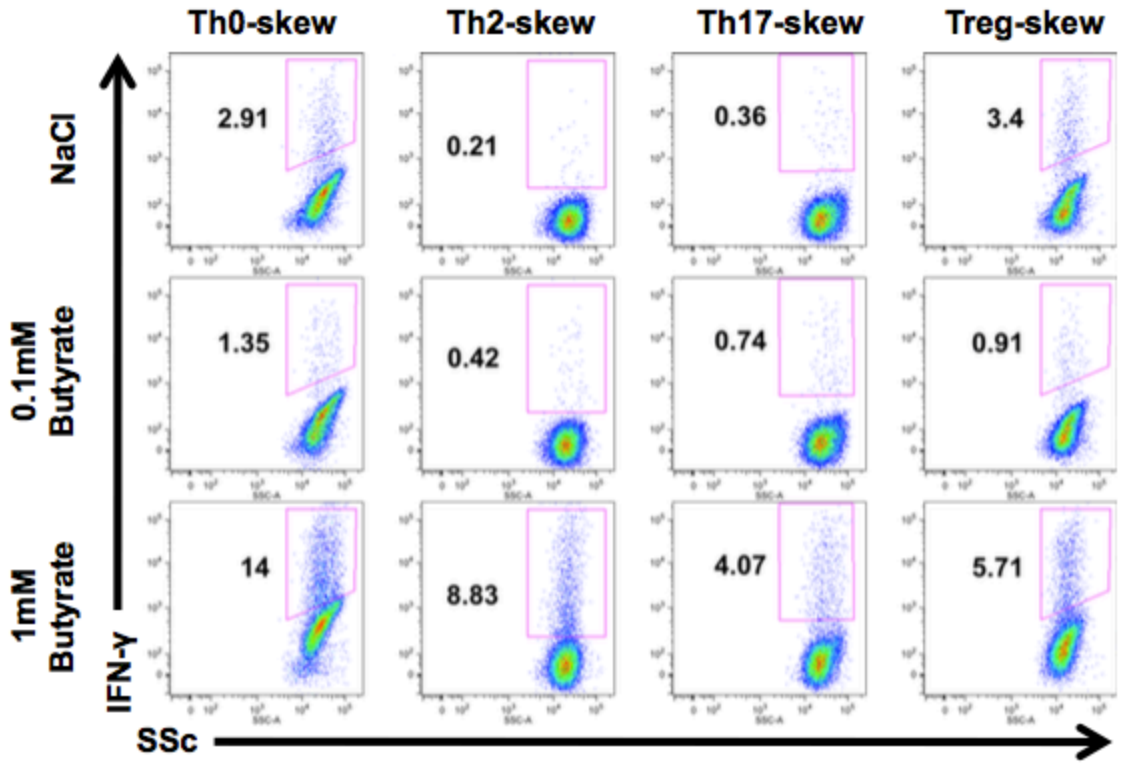

Figure 49. Butyrate does not cause toxic effects during T cell differentiation.

CD4+ CD25- T cells sorted to high purity were cultured with TCR-crosslinking for 72 hours in the presence of Th0-, Th2-, Th17-, or Treg- skew cytokines and indicated concentration butyrate or NaCl control. At the end of culture, cells were restimulated with PMA and ionomycin in the presence of golgi plug followed by intracellular staining for intracellular and PBSE Live/dead discrimination dye. Data shown is from PBSE negative population.