MICROBIOTA-MEDIATED PREVENTION OF PRO-ATHEROGENIC FOAM CELL FORMATION- UNCOUPLING AUTOPHAGIC, METABOLIC, AND INFLAMMATORY MECHANISMS

A Dissertation

by

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ABSTRACT

A significant hallmark of the chronic inflammatory disease, atherosclerosis, is the accumulation of pro-inflammatory M1-like Macrophages (Macs) in coronary arteries that respond to pro-atherogenic stimuli (such as free fatty acids (FFAs) and oxidized LDLs (oxLDLs) and fail to digest lipids that contribute to the formation of foam cells in atherosclerotic plaques. Mechanisms that reduce Mac/ dendritic cells (DCs) inflammation, increase lipid degradation, and prevent foam cell formation are likely to decrease atherosclerosis progression.

The cell recycling program autophagy is critical to prevent lipotoxicity and reduce foam cell formation in atherosclerotic Macs and DCs. Increased autophagy helps regulate lipid metabolism and prevent the development of atherosclerosis. Mechanisms that regulate autophagy in Macs will likely reveal targets for the prevention of atherosclerosis.

Disrupted microbiota is linked with multiple diseases, including cardiovascular disease (CVD), and microbiota-derived TMAO metabolite production is one deleterious mechanism that promotes atherosclerosis. However, further research is necessary to identify new microbiota metabolites and determine their positive or negative role in CVD. Our overall goal in this project was to discuss the role of microbiota in promoting health and disease by the production of specific beneficial and detrimental metabolites that impact the development of atherosclerosis by jointly regulating chronic endotoxemic and lipotoxic inflammation, metabolic disruption in innate immune cells.

We have investigated tryptophan-derived microbiota metabolites, as novel aryl hydrocarbon receptor (AhR) ligands with immunomodulatory properties for APCs. In particular, indole metabolite. Indole is abundant in the GI tract and can enter into the blood circulation. Our objective in this study was to study the molecular mechanisms whereby microbiota regulates autophagy in Macs and to determine the functional consequences of microbiota-induced autophagy on innate immunity in response to atherogenic stimuli.

DEDICATION

To my parents, husband and my sister who gave me their love, support and encouragement all the time.

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CONTRIBUTORS AND FUNDING SOURCES

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NOMENCLATURE

CVD	Cardio-vascular disease
Mac	Macrophage
DC	Dendritic cell
APC	Antigen-presenting cell
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived Mac
WT	Wild Type
AhR-KO	Aryl hydrocarbon Receptor Knock Out
TLR	Toll like receptor
LPS	Lipopolysaccharide
HFD	High fat diet
FA	Fatty Acid
SFA	Saturated fatty acid
Treg	Regulatory CD4+ T helper cell
AhR	Aryl hydrocarbon receptor
Trp	Tryptophan
SCFAs	Short-Chain Fatty Acids
AhR	Aryl Hydrocarbon Receptor
DMF	Dimethylformamide
D	DMF
I	Indole

TMAO	Trimethylamine-N-oxide
TMA	Trimethylamine
I3S, IS	Indoxyl Sulfate
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
Ox-LDL	Oxidized LDL
SCFAs	Short chain fatty acids
Th17	CD4+ T Helper Type 17

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Cardiovascular disease (CVD) is a complex human disease that may restrict blood flow in the heart and blood vessels (1). Atherosclerosis, an excess buildup of arterial plaque (atheroma) along the arterial wall, is the primary driver of CVD (2). The plaque is composed of lipids (cholesterol and fatty acids), debris, fibrotic material, and Macrophages (Macs) and dendritic cells (DCs), among other host immune cells (3). Within the atherosclerotic plaques, Macs have a pro-inflammatory predisposition and attempt to ingest and degrade debris and lipids that contribute to the formation of foam cells - all of which are associated with increased plaque formation and adverse effects for the host blood flow (4).

Autophagy is a major and conserved mechanism in cell biology that performs housekeeping functions and promotes cell survival during nutrient starvation and is an important mechanism that promotes not only homeostasis but also inflammation (i.e., regulates NLRP3-inflammasome) and cellular metabolism (i.e., regulates mitochondrial and cellular energetics) (5). Reports show that dysregulated or non-functioning autophagy leads to altered gut immune and metabolic homeostasis. Recently, several notable studies have determined that autophagy is critical to prevent lipo-toxicity and reduce foam cell formation in atherosclerotic Macs (6). Increased autophagy helps regulate lipid metabolism and prevent the development of atherosclerosis (6). Therefore, a deeper understanding of the mechanisms that regulate autophagy in Macs and DCs will likely reveal potential targets for the prevention of atherosclerosis.

There is a high level of interest in identifying metabolites of endogenous or digested dietary compounds generated by the GI tract microbiota and determining their functions in health or

disease (7). A study by Wikoff et al. demonstrated that tryptophan-derived compounds were depleted in the GI tract and the circulation of germ-free mice, indicating they are dependent on the microbiota. In particular, indole was not observed in cecal tissue from germ-free mice (8).

There is now an undeniable appreciation that the microbiota is an essential partner in overall gut homeostasis and host health. Furthermore, when the microbiota is disturbed/perturbed from environmental or dietary stresses (referred to as dysbiosis), this can lead to increased host inflammation and altered metabolism (9). There is a wealth of compelling evidence that the microbiota is linked with multiple complex diseases, including CVD (10); however, our understanding of the microbiota mechanisms that mediate beneficial or harmful effects on CVD are limited. Perhaps the most specific example of the link between the microbiota and CVD is the microbiota-dependent production of trimethylamine-N-oxide (TMAO), from diets rich in phosphatidylcholine, choline, and L-carnitine, that is associated with a significant increased risk of atherosclerosis (11). Although a number of microbiota-derived metabolites have been identified and studied, the full array of activities for most individual metabolites has not been completely established, and further research is needed to better understand the natural breadth of metabolites, the microbe(s) that produce them, their cellular and molecular targets, and their role in health and disease – this is especially true in the context of CVD. Thus, in this project, we overall discuss that the microbiota promotes health and disease by the production of specific beneficial (e.g., SCFAs, H2S, and others) and detrimental (e.g., TMA/TMAO, γ-butyrobetaine, etc.) metabolites (12), and impacts the development of atherosclerosis in obese patients by jointly regulating chronic endotoxemic and lipotoxic inflammation and metabolic disruption.

In line with published data, our team investigates the impact of the microbiota as a rich source of potent immunomodulatory metabolites derived from tryptophan (Trp) (13). In particular,

the primary microbiota-derived Trp-metabolite indole (C8H7N) that is a potent endogenous ligand for the aryl hydrocarbon receptor (AhR), regulates gut inflammation, and microbiota dysbiosis (14).

In addition, we investigate the role of microbiota metabolites in the HFD-fed animal and its role in the regulation of Macs inflammation, metabolic pathways, and autophagy in a manner that indicates these metabolites may play a role in the development of complex inflammatory diseases such as atherosclerosis. Therefore, we specifically study the inflammatory and metabolic responses of pro-atherogenic phagocytes exposed to lipotoxic compounds and their regulation by the microbiota-derived or dependent metabolites.

Microbiota Metabolites in Health & Disease

The microbiota in the GI tract, whose microbiome has 70 – 140 times more genes than its host, can perform complex biosynthetic functions and bio-transformations that may yield beneficial or deleterious bioactive compounds (15).

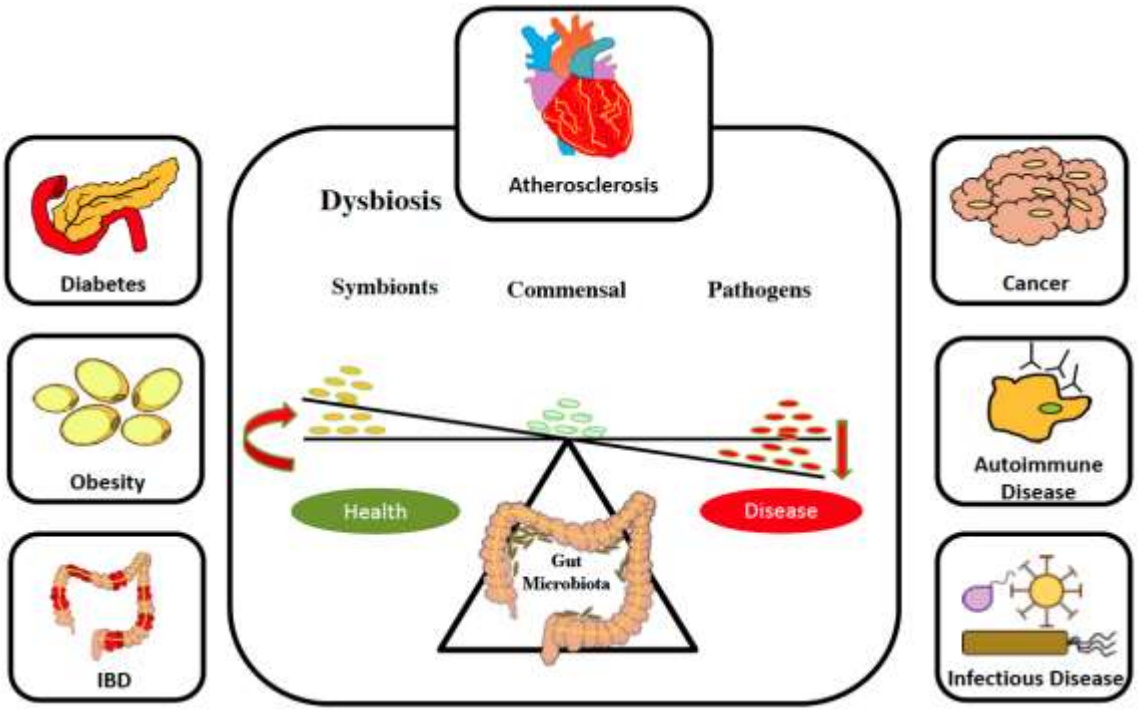

Figure 1 The microbiota promotes overall health and, when perturbed, cause disease.

The balance between health and disease in many ways is regulated by microbiota. Microbiota in equilibrium is linked with health, and when it is perturbed, it leads to disease.

Recent studies have clearly demonstrated that this diverse panel of metabolites is not simply by-products but are active molecular determinants of cellular behavior and responses in vivo (16). Therefore, there is a high level of interest in identifying metabolites or derivatives of endogenous or digested dietary compounds generated by the GI tract microbiota, and in determining their functions in health or disease (Figure 1). Although several microbiota-derived

metabolites have been identified and studied, the full array of activities for most individual metabolites has not been completely established. However, other than SCFAs, and in contrast to pro-atherogenic TMAO (17), no clear studies have reported microbiota metabolites with direct beneficial or anti-atherogenic functions in reducing atherosclerosis. Disrupted microbiota is linked with multiple diseases, including cardiovascular disease (CVD), and microbiota-derived TMAO metabolite production is a deleterious mechanism that promotes atherosclerosis (18). However, further research is necessary to identify new microbiota metabolites and to determine their positive or negative role in the context of CVD.

Microbiota in Atherosclerosis

The microbiota is recognized for its role in the production of beneficial SCFAs and its functions in regulating our susceptibility to complex diseases such as atherosclerosis. A link between the microbiota and atherosclerosis emerged from studies on TMAO, produced from microbiota trimethylamine (TMA) (17); yet no reports of Trp metabolites having a role atherosclerosis have emerged.

A significant hallmark of the chronic inflammatory disease, atherosclerosis, is accumulation of pro-inflammatory M1-like Macs and dendritic cells (DCs) in coronary arteries that respond to pro-atherogenic stimuli (such as free fatty acids (FFAs) and oxidized LDLs (oxLDLs) and fail to digest lipids that contribute to the formation of foam cells in atherosclerotic plaques (19). Mechanisms that reduce Mac/DC inflammation, increase lipid degradation, and prevent foam cell formation are likely to decrease progression of atherosclerosis.

There is a high level of evidence that microbiome, microbial metabolism, and microbiota derived, or dependent nutritional metabolites have a big contribution in atherosclerosis (20). The mechanistic link between Gut microbiota dependent/derived metabolites are still undefined.

Indole, TMAO and Indoxyl sulfate are among the Microbiome derived/dependent metabolites that have been reported to have roles in regulation of atherosclerosis (21).

TMAO (Trimethylamine-N-Oxide)

TMAO is a microbial dependent metabolite. It is a byproduct of microbial metabolism of L-carnitine and choline (found in eggs, meat, and fish) in the gut, and it has a direct correlation with atherosclerosis (22). After metabolizing carnitine and choline to TMA (trimethylamine), through the bacteria, TMA gets absorbed from the gut to the circulation (23). Then, in the liver, via an enzyme named Flavin mono-oxygenase, TMA gets oxidized into TMAO. Other than atherosclerosis, plasma levels of TMAO increase in patients with Chronic Kidney diseases (CKD), diabetes (24). The reason for the TMAO increase in these diseases is still unknown. However, studies suggest that reduced clearance of TMAO by Kidneys in CKD or increased levels of TMAO metabolism by bacteria in the gut can be among the possible reasons (25). Some data suggest that dysbiosis has a direct link with increased serum levels of TMAO. In these patients, the relative abundance of dominant bacteria changes, and consequently, the gene expression of the enzyme that leads to TMAO production will change and increase (26).

Rodent studies also show that TMAO enhances atherosclerosis prevalence and risk factors through increasing the expression of scavenger receptors and reducing cholesterol efflux in Macs and consequently increased levels of foam cell formation of the aforementioned Macs (27). This proatherogenic metabolite has a positive correlation with lesion formation, development, and size of the atherosclerotic plaque in arteries (28).

Atherosclerosis starts with the accumulation of foam cells in the arteries. Monocytes that are in the circulation will penetrate to the arteries that have lesions, and in the arteries, they will transform into Macs, and by phagocyting modified cholesterol and lipoproteins, these Macs will

turn into foam cells (Figure 2). Accumulation of foam cells under the arteries endothelial cells will form plaques. Rupture of the plaque can block arteries and cause a stroke (29).

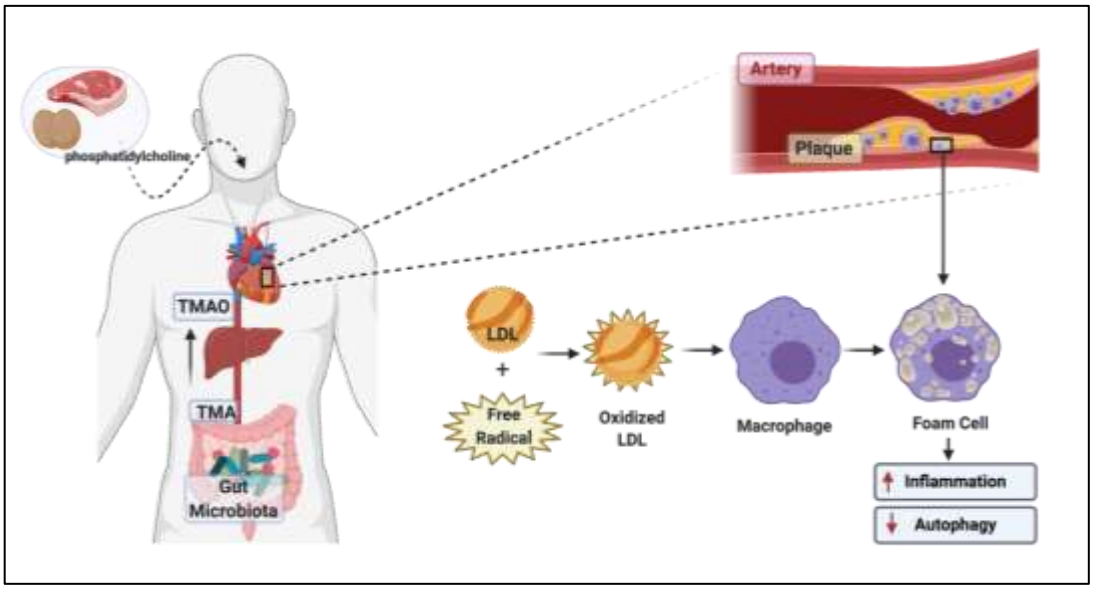

Figure 2. Role of trimethylamine N-oxide in atherosclerosis.

Schematic pathway of phosphatidylcholine transformation to TMA and TMAO (trimethylamine N-oxide) via the gut microbiota. Dietary intake of foods like red meat and egg can alter the composition of gut microbiota. On the other hand, it can result in increased TMA production levels, subsequently leading to increased TMAO synthesis in the liver and, eventually, to elevated levels of oxidized LDLs and promoted plaque formation. Accumulated foam cells in the plaques are lipid-laden Macs that have ingested modified lipoprotein s and have a foamy appearance. Inflammatory Macs that turn into foam cells are highly inflammatory and have downregulated autophagy levels.

Macs can regulate lipoprotein metabolism, and they are the key cells involved in atherosclerosis because they are the origin of the foam cells. So, the migration of the Macs to the plaque areas and their foam cell transformation mechanism and pathways involved in these mechanisms are very important to study. There are pieces of evidence that are suggesting that TMAO is involved in this mechanism (30).

When the LDLs or low-density lipoproteins get oxidized with free radicals in the arterial walls, the Macs will be triggered to phagocyte these modified lipoproteins through increasing the expression of scavenger receptors such as CD36 and SRA-1 that have high sensitivity to modified lipoproteins (31). TMAO, based on the studies, can upregulate the expression of these scavenger receptors and induce the uptake of these modified LDLs by Macs (32). TMAO can increase the migration of Macs and promote the expression of inflammatory cytokines such as IL-6 and TNF (33). Based on my studies, TMAO can polarize and activate BMDMs towards the inflammatory state, and these inflammatory Macs have a higher contribution to penetration and foam cell formation in the plaques.

Indoxyl sulfate (I3S)

Indoxyl sulfate, AKA, 3-indoxyl sulfate, and 3-indoxylsulfuric acid (I3S) Is a bacterial metabolism byproduct of the dietary nutrients. I3S plays a role as a uremic toxin and a cardiotoxin (34). A microbial enzyme, named tryptophanase, can catabolize the tryptophan to indole. Then the indole gets absorbed and converted into indoxyl sulfate in the liver. Dysbiosis and epigenetic alterations of the gut microbiome, changes the amino acid metabolism and favors in the enhanced levels of I3S in the serum (Figure 3) (35). Also, the amount of tryptophanase expression in microbiome increases to facilitate the production of Indoxyl sulfate in CKD and CVD patients (36). The prevalence of atherosclerosis is higher in CKD patients (37). And based on the epidemiology studies, a high risk of atherosclerosis in CKD patients is beyond traditional risk factors, indicating the possible role of microbiota in regulating the disease.

Plasma concentration of I3S increases in patients with atherosclerosis and CKD (38). I3S has a high affinity to proteins, and therefore it is hard for kidneys to remove it (39). In vitro studies show that indoxyl sulfate can enhance leukocyte activation and increase their adhesion to

endothelial cells and eventually cause elevated levels of oxidative stress and inflammation (40). Furthermore, it is hypothesized that indoxyl sulfate reduces the cholesterol efflux in Macs and induces the foam cell formation (41). I3S is also related to glucose intolerance by reducing GLUT-1 expression and the LXR signaling pathway in the liver (42). Increased levels of atherosclerosis are reflected by increased levels of inflammatory Macs. My experiments show that indoxyl sulfate increases the expression of proinflammatory markers in BMDMs.

Proteomics studies indicate activation of some pathways via I3S in Macs, such as ubiquitin-proteasome pathway and Notch signaling (43). Some studies mention that membrane transport proteins such as OATP2B1 regulates the uptake of I3S in Macs (44). I3S is an agonist for AhR, and it can increase vascular smooth muscle cell proliferation through AhR and activating NF-κB signaling (45). Furthermore, I3S increases ROS production (46).

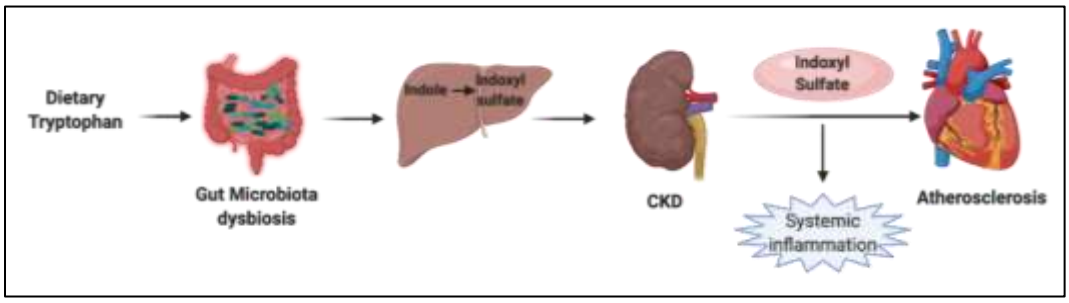

Figure 3. Effect of Indoxyl sulfate in atherosclerosis.

Schematic presentation of the Indoxyl sulfate pathway link to atherosclerosis. Tryptophan is metabolized by gut microbiota to indole, and indole is absorbed into circulation. In the liver, indole is metabolized to indoxyl sulfate. In chronic kidney disease and dysbiosis conditions, kidneys are incapable of clearing indoxyl sulfate. This results in the accumulation of indoxyl sulfate. Systemic inflammation caused by indoxyl sulfate can cause coronary calcification and chronic abnormalities in CVD and eventually cause atherosclerosis.

Indole

Indole is a gut microbiota-derived, Tryptophan catabolite. It is produced after tryptophan metabolism by tryptophan lyase (tnaA) enzyme (Figure 4) (47). Indole is an agonist for the aryl hydrocarbon receptor (AhR). There is a high concentration of indole in the GI tract, and it can enter the blood circulation (48). Indole is detected in human and mouse luminal contents at 0.1 to 4 mM concentrations and around 0.1 to 10 μM in the circulation (49). Indole is a very small molecule, and it easily diffusible and can have direct contact with immune cells (50).

Our lab and other research have indicated that indole has anti-inflammatory effects (51). Studies show that plasma levels of indole and Indole derivatives are negatively related to advanced atherosclerosis (52). Indole is produced by a variety of gram-negative and gram-positive bacteria species such as E. coli, Bacteroides and Clostridium (53). My studies indicate that indole can reduce the foam cell formation of Macs through different mechanisms and can be a good endogenous metabolite candidate for reducing atherosclerosis progression or onset. Our overall findings show that indole has anti-inflammatory properties, activates autophagy, and regulates the metabolism towards the reduction of lipid droplet accumulation in Macs that are the key target cells in atherosclerosis. My studies show that indole has similar effects to rapamycin. Other studies in our lab show that indole conditioned APCs can induce anti-inflammatory phenotypes in naïve T cells. We and other labs' in vitro studies have also indicated that other than immunomodulatory effects, indole can promote intestinal epithelial cells' health in humans and rodents by preventing colitis induced by dextran sulfate sodium (DSS) and strengthening epithelial barrier (54). In line with our studies, some observations suggested the important positive role of indoles supplementation to anti-inflammatory drugs (55).

Gut bacteria genomic information of the human microbiome suggests that tryptophan metabolites are the most important bioactive microbiota metabolites (56). Studies demonstrate that in atherosclerotic patients, microbiome tryptophan synthesis and consequently, plasma levels of tryptophan metabolites are reduced (57). There are some tryptophan derivatives, such as indoxyl sulfate or indole acetate, that their increased levels have been reported to have a direct correlation with CVD and other diseases (58). This suggests the importance and sensitivity of the equilibrium of microbial tryptophan metabolites in the host's gut and overall health. Molecular targets of tryptophan derivatives are still unknown, but some indoles have been known to have modulatory effects through AhR or aryl hydrocarbon receptor (59).

Some studies propose the development of antibiotics to control the metabolic changes in atherosclerosis (57).

Studying the cross-talk between metabolic changes, immune cells, and molecular targets is very important in atherosclerosis research.

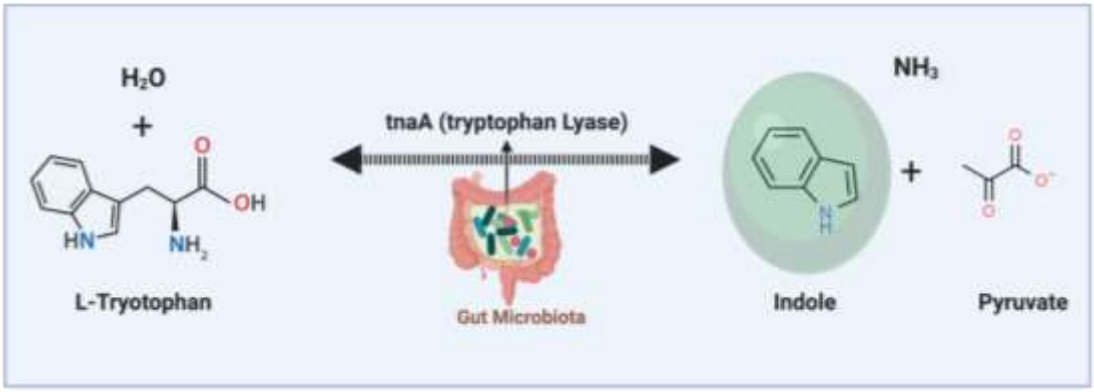

Figure 4. Indole is a gut microbiota-derived metabolite and is selectively produced by the microbiome.

Indole Is produced from tryptophan amino acid through the action of the tryptophan lyase enzyme. Indole is an aromatic heterocyclic organic molecule and has a bicyclic structure. Indole has anti-

inflammatory regulatory effects on intestinal epithelial cells and Immune cells in the gut and distal locations in the body.

Aryl hydrocarbon Receptor (AhR)

Aryl Hydrocarbon Receptor or AhR is a transcription factor. AhR activates through various endogenous and exogenous polycyclic aromatic hydrocarbon ligands (60). It is involved in different cellular activities such as cell differentiation and proliferation, cytokine production, and reactions related to environmental toxins (61). The potential role of AhR has been suggested concerning the gut and immune system. AhR has roles in the regulation of the gut's intraepithelial lymphocytes (IELs) and innate lymphoid cells (ILC) (62). AhR can regulate the induction of T-reg and Th17 (63).

The cross-talk between intestinal microbiota and immunity and its relationship with AhR in APCs has been less characterized and requires more investigation. AhR can detect environmental signals; it is also present in immune cells (64). That's why some consider it a candidate pattern recognition receptor sensor for immune responses driven by nutritional and microbial gut metabolites (65).

We have identified microbiota metabolites derived from essential amino acids that have regulatory properties for host cell functions. We also identified that these metabolites derived from tryptophan are agonists for AhR. Based on the cell type, indole may regulate the cell, dependent, or independent of AhR. So far, in our studies, indole regulated DC and Macs in an AhR independent manner. Based on our overall hypothesis, microbiota regulates the host through metabolites, and there are already examples of known metabolites. But because of the very important role of the tryptophan derived microbial metabolites in immunoregulation, we focused on indoles that are ligands for AhR. The AhR properties have been studied. AhR has first identified

as a receptor for industrial toxin n-dioxin (66). Now, AhR is a very important mediator of immune cell activity, particularly in the GI tract (67). But it identified to be very important to non-endogenous AhR ligands in differentiating T-reg and Th17 (63). AhR is also called a ligand-activated transcription factor. It exists in the cytosol, binds to the ligand, and then translocates to the nucleus via a nuclear translocator to act as a transcription factor (68). Knowing the effects and its role in homeostasis, particularly on innate lymphoid cells, it is becoming clear that microbiota's act through the AhR is one of the mechanisms that can mediate the immune cell cross-talk. Our studies of indole as a ligand of immunoregulatory AhR indicates an important mechanism of action. Based on our observations, indole, and 5-Hydroxyindole (5HI) regulate the cell in an AhR dependent manner in T-cells. But in contrast, when we used AhR-KO Macs or DCs and did the same experiments with indole and 5HI, indole was still capable of inducing its activity, independent of the AhR.

Based on a study that was aiming to detect a panel for AhR ligands in BMDCs, some ligands like I3C (indole 3-carbinol), FICZ (6 formyl indolo carbazole) has been among them that induced pro-inflammatory effects in LPS induced APCs which is in contrast with the studies that suggest AhR activation has immunomodulatory effects (69). For example, 4-n-nonylphenol, which is an agonist for AhR, can induce T-regs (70). The published data suggest that AhR signaling in monocyte-derived-Macs can have effects on their function (71). But a detailed investigation of ligands and their functional dependency on AhR is necessary for unraveling the role of AhR in regulating Macs through endogenous intestinal ligands.

Studies highlight that expression of AhR is related to atherosclerosis (72), but the effects of AhR activation is dependent on the agonists, species, and cell type. For example, in ApoE-KO mice, increased AhR is linked to increased signs of atherosclerosis, but on the other hand,

activation of AhR through indoles has modulatory effects on the reduction of CVD (72,73). Some studies highlight the beneficial role of specific indoles in prevention of atherosclerosis (74).

In summary, data regarding AhR in atherosclerosis shows that depending on the different circumstances, AhR activation can have positive or negative effects. Since the role of AhR is different in species, driven data from rodent studies may not be conclusive enough for humans.

Autophagy in Cellular Homeostasis

Autophagy is a "self-eating" process, in which, cytosolic content and organelles are sequestered within a single or double membrane compartment (called the autophagosome), targeted to the lysosome (forming an autophagolysosome) and rapidly degraded (75). The membrane dynamics of the autophagic process involve various protein complexes required for nucleation and elongation of the autophagosome. Initial steps of the canonical autophagosome biogenesis pathway require the activity of class-III-phosphatidylinositol 3-kinase (PI3K) Vps34 (76). Inhibitors of Vps34, such as methyladenine (3-MA), prevent autophagy induction and therefore provide useful pharmacological tools for interrogating autophagic processes (77). The elongation of the autophagosomal membrane requires the activities Atg9— which shuttles disparate intracellular membranes to sites of autophagosome elongation— and two ubiquitin-like conjugation systems (78). In the Atg5-Atg12 system, Atg12 is conjugated to Atg5 in a process that requires Atg7 (ubiquitin-activating-enzyme (E1)-like) and Atg10 (ubiquitin-conjugating-enzyme (E2)-like) (79). The Atg5-Atg12 complex then interacts with Atg16 to drive the ubiquitin-like conjugation of LC3-I (the mammalian ortholog of Atg8) to phosphatidylethanolamine (PE), resulting in the formation of membrane-associated LC3-II (Figure 5) (80). As might be expected for a process that is central to eukaryotic cell function, autophagy is subject to strict control by diverse signaling systems.

For example, the AMPK/ULK1 and mTOR signaling complexes, respectively, positively and negatively regulate the induction of canonical autophagy (81). Finally, it should be noted that autophagic processes that are independent of the Atg5-Atg12 system have been described (82). Recently, it has been recognized that autophagy plays a prominent role in intestinal homeostasis and innate immunity, where it serves to capture and clear invading intracellular pathogens (83). In addition, autophagy plays an important role in antigen presentation and can affect the peptide loading of MHC class I and II molecules (84).

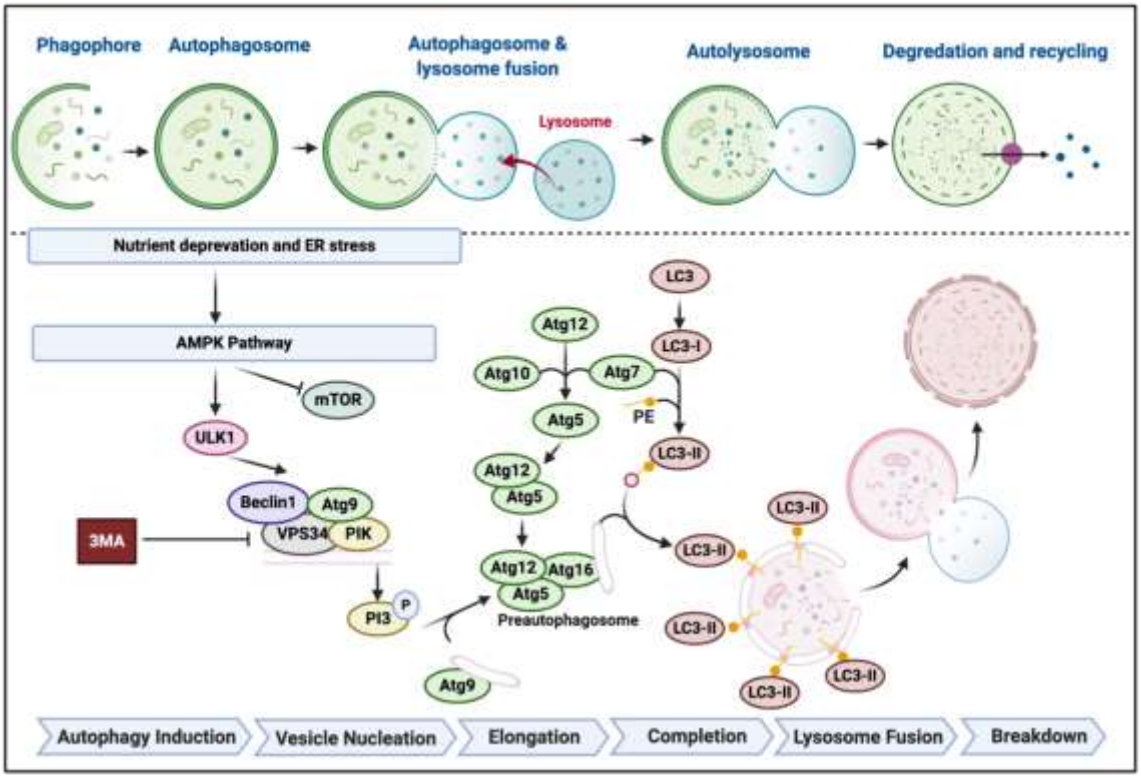

Figure 5. Autophagy in Cellular Homeostasis.

The membrane dynamics of the autophagy involve protein complexes required for nucleation and elongation of the autophagosome. Initial steps of the autophagosome biogenesis pathway need the activity of class-III-phosphatidylinositol 3-kinase (PI3K) Vps34. Inhibitors of Vps34, such as methyladenine (3-MA), prevent autophagy induction. The elongation of the autophagosomal membrane requires the activities Atg9, which shuttles intracellular membranes to autophagosome

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Mac and DC Autophagy and Inflammation in Atherosclerosis

M1-like inflammatory and metabolically active (glycolysis) Macs and DCs are major cellular participants in plaque formation (85). Macs numbers in atherosclerotic mouse aortae increase up to 20- fold during atherogenesis (86). Mac autophagy is an important cellular mechanism for optimum antigen/particulate degradation and reduced cellular inflammation (Figure 6). Most Macs in atherosclerotic plaques are filled with cytosolic lipid droplets (LDs) (87). In advanced atherosclerosis, Macs and DCs provide inflammatory signals and fail to effectively clear lipids and debris (88).

The cell recycling program autophagy is critical to prevent lipotoxicity and reduce foam cell formation in atherosclerotic Macs, and increased autophagy helps regulate lipid metabolism and prevent the development of atherosclerosis (89). Mechanisms that regulate autophagy in Macs and DCs will likely reveal targets for the prevention of atherosclerosis. Recently articles have established that phagocyte autophagy, when strongly active, is a critical cellular process that serves to prevent and eliminate plaque buildup in animal models of atherosclerosis (90).

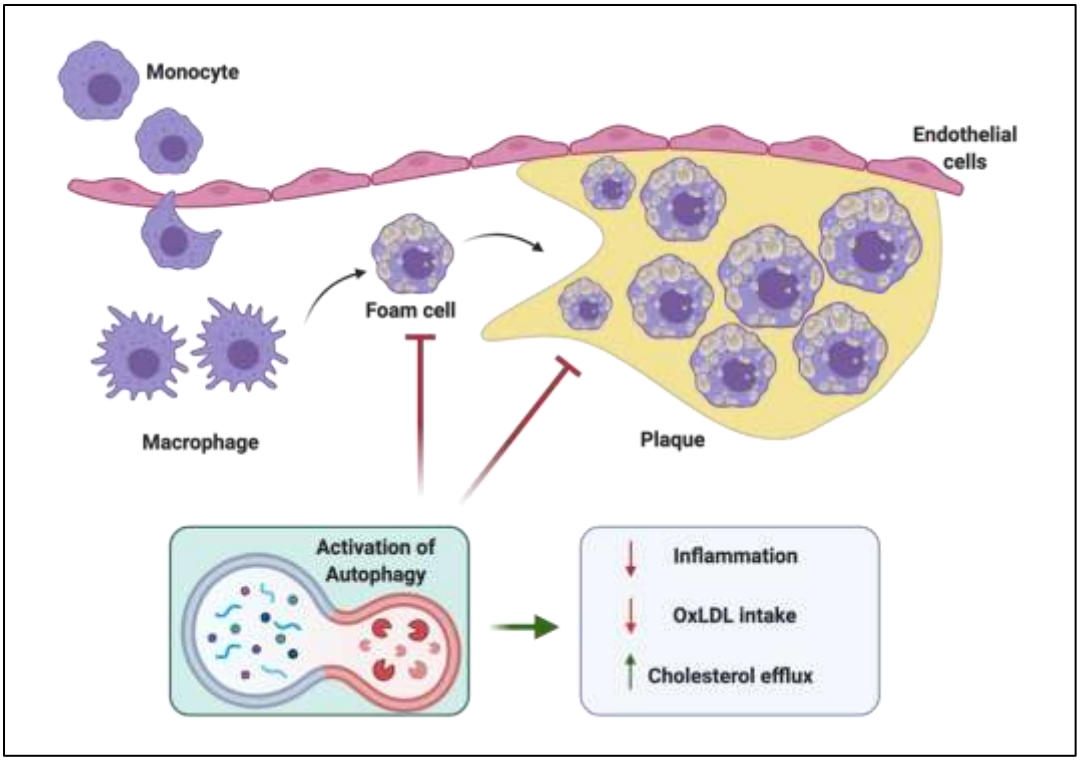


Figure 6. Role of active autophagy in Macs foam cell formation.

In atherosclerosis, when there is a damage in the endothelium (inner wall of an artery), monocytes in the circulation, travel to the damage site, engulf fats, modify LDLs, and form plaques. Active autophagy regulates cholesterol efflux, inhibit inflammation, and reduce ox-LDL intake in Macs and foam cells and finally limit the atherosclerotic plaque buildup.

Cardio-metabolism, endotoxemia and Inflammation

Lipopolysaccharides (LPS), AKA, bacterial endotoxins, or Lipoglycans) are macromolecules composed of an O-antigen containing polysaccharide and a lipid that are covalently bonded. The outer cell membrane of the gram-negative bacteria has conserved components of LPS (91). LPS in gram-negative bacteria can lead to activation of Innate Immunity and, consequently, the onset of inflammatory reactions (92). High levels of LPS (because of gram-negative bacteria) can cause sepsis both in humans and rodents (93). “Metabolic endotoxemia”, happens when there is a low but constant level of LPS in blood circulation that can cause innate

immune responses and slight consistent inflammation in the circulation without signs of significant infection (94).

Other than sepsis, there are other diseases that are caused by metabolic, inflammatory dysregulations. Examples of metabolic, inflammatory diseases are Insulin resistance (IR), Type two diabetes, and atherosclerosis (95). Many factors in obesity (such as high levels of cholesterol, saturated fatty acids, endotoxins, etc.) can raise the levels of inflammation, reverse cholesterol transport (RCT), and reduce insulin signaling (96). Macs are the most important cells that are involved in the process of upregulation and downregulation of metabolic-inflammatory responses through induction of inflammatory and anti-inflammatory metabolic pathways in response to an overabundance of lipids from adipocytes; such as saturated fatty acids (particularly palmitate) and circulating modified lipids that are pro-inflammatory (97). AMPK (AMP-Activated protein kinase) and PPARs (Peroxisome proliferator-activated receptors) are important regulators of metabolic and inflammatory mechanisms through modulating cellular homeostasis (98). Palmitate is a known saturated fatty acid that can cause metabolic inflammation through NLRP3 or (NOD)-like receptor protein3 inflammasome, which can inhibit AMPK activation (97,98). Unsaturated fatty acids normally do not cause metabolic inflammation (99).

Based on many studies, high levels of LPS in the serum directly affect the onset or exacerbation of CVD, especially atherosclerosis (100). Experiments in rodents have indicated that endotoxins can accelerate the incidence and progression of atherosclerosis (101). LPS receptor is Toll-Like receptor-4 or TLR-4. Based on the published experiments, mice that are deficient for TLR-4 are more resistant to the development or progression of atherosclerosis (102). It is hypothesized that when there is no infection, but endotoxin is present, LPS from intestinal gram-negative bacteria or from nutrients can translocate from intestinal lumen to the circulation (103).

Furthermore, in rodents and humans, increased levels of endotoxemia have a direct correlation with increased levels of dietary fat uptake (94).

Permeability of intestinal lumen plays an important role in the severity of the endotoxemia, because of the high density of endotoxins in the intestine compared to the plasma (104). Increased levels of plasma endotoxins have found in humans with CVD, and they indicate signs of luminal permeability problems (100). Microbiota and the metabolites that are being produced by gut microbiome play an important role in the permeability of intestinal lumen (105).

Mac reprogramming and activation states

Macs are one of the key cells of the innate immune system. They are involved in clearing infectious diseases, antigen presentation, regulating inflammation, and phagocytosis. Furthermore, Macs are involved in the regulation of other immune cells by releasing various cytokines (106). There is a big spectrum of Mac phenotypes (107). Depending on the signals that Macs are exposed to, they can get polarized towards different activated states. Macs manifest metabolic alterations in different diseases, especially in atherosclerotic plaques (107). Polarized Macs can reprogram their glycolytic metabolism, lipid metabolism, amino acid metabolism, energy metabolism as well as mitochondrial oxidative phosphorylation (OXPHOS). Classically activated M1 Macs and alternatively activated M2 Macs are the two most studied phenotypes of Macs. Non-activated quiescent Macs are referred to as M0 Macs (108).

Polarization of M0 Macs towards an M1 phenotype can be achieved by stimulation of bacterial lipopolysaccharide (LPS), via the PI3K-AKT-mTOR-HIF1a signaling pathway, in the presence of inflammatory cytokines, and in the presence or absence of Interferon-gamma (IFN-γ), and/or tumor necrosis factor (TNF) (109). M1 Macs have increased levels of glycolysis

metabolism, decreased levels of oxidative phosphorylation (OXPHOS), and produce inflammatory cytokines involved in infectious and inflammatory diseases (108).

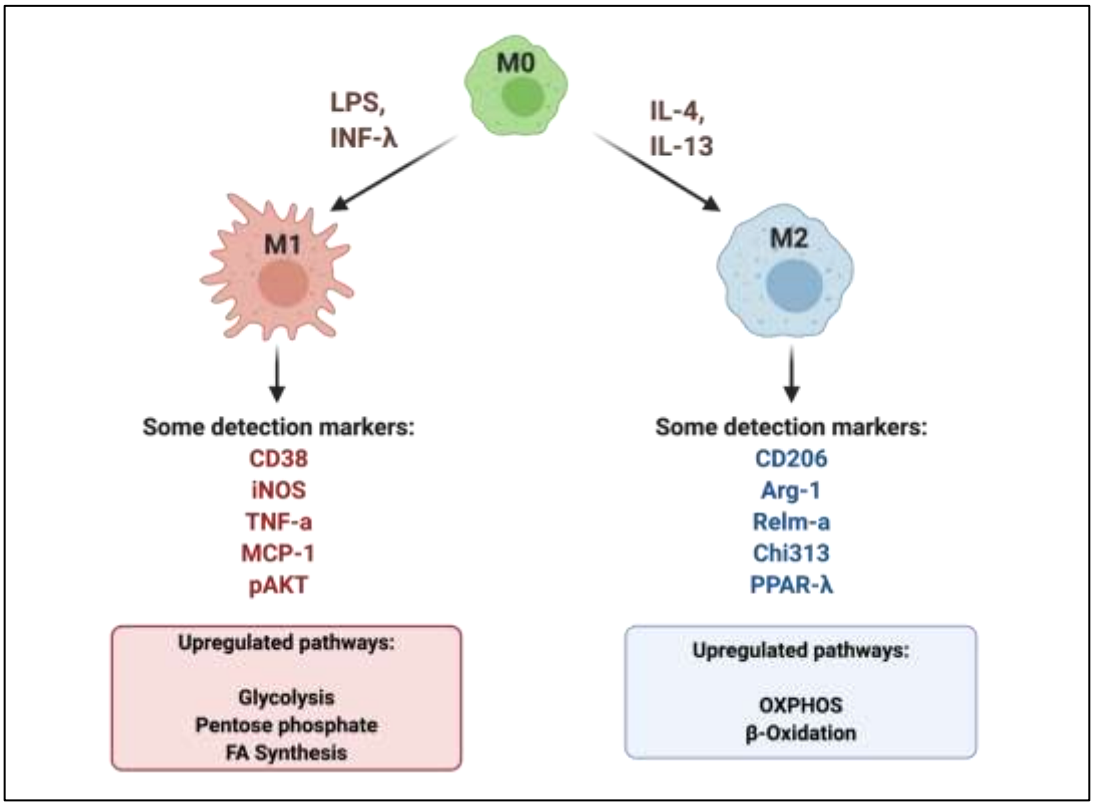


Figure 7. M1 and M2 polarization of Macs.

Depending on the polarization signals, naïve Macs can get polarized towards M1 (LPS, INFλ skews) or M2 (IL-4, IL-13 skews) like Macs. There are several surfaces, intracellular or intranuclear markers for the detection of Macs phenotype. Other than recognition markers, some specific pathways get upregulated and downregulated in M1 and M2 Macs. For example, glycolysis, pentose phosphate pathway, and fatty acid synthesis get upregulated in M1 Macs. Oxidative phosphorylation and β-oxidation are pathways that get upregulated in M2 Macs. PPAR, peroxisome proliferator-activated receptor; CD38, Cluster of differentiation 38, one of the main cellular NADases in mammalian tissues; MCP-1, The monocyte chemoattractant protein-1; iNOS, Inducible nitric oxide synthase; STAT3, signal transducer and activator of transcription 3; Arg-1, Arginase 1; Resistin-like molecule alpha; Egr2, Early growth response protein 2; CD206, mannose

receptor, and C-type lectin; Chi313, chitinase-like lectin; AKT, Protein kinase B (PKB); TNF, Tumor necrosis factor.

On the other hand, polarization towards M2 Macs can be activated by Interleukin (IL)-4, via the JAK-STAT, PPAR, and AMPK pathways, or by (IL)-13 and Transforming growth factor- β (TGF-β) (109). M2 Macs have anti-parasitic and tissue repair activities as well as increased levels of OXPHOS, contrary to M1 Macs (110). Surface and intracellular markers, as well as the genes that become upregulated and downregulated in polarized Macs, have been discussed in many reviews (111). Macs in humans and mice are identified by expression of different surface markers, which most of them are overlapping, and very few of them are specific for a polarized phenotype. For example, CD38 and MCP-1 are two M1 polarization markers, and CD 206 and Arg-1 are common M2 markers for characterizing mice polarized Macs. And because of the possibility of overlapping phenotypes, it is always suggested to test the functional features of polarized Macs along with surface markers (Figure 7) (111,112).

Two of the main energy production pathways in the cells are OXPHOS and glycolysis. Based on their microenvironment, Macs can choose to use either of these pathways or, in some cases, switch from one pathway to the other or use both pathways (113).

Mac polarization in atherosclerotic plaques

Local inflammatory responses in atherosclerosis can activate different cells that are in the atherosclerotic lesion area (114). Increased levels of modified LDLs or Oxidized LDLs can cause the migration of a significant number of monocytes into the atherosclerotic plaque area beneath endothelial cells in the arterial wall (115). The inflammatory microenvironment of the lesion will differentiate the monocyte to Macs. The Macs will start to phagocyte the modified lipoproteins in

their environment and transform into foam cells and eventually, retain in the plaques (116). These Macs will release inflammatory cytokines and induce more inflammation. Consequently, more monocytes will be recruited to the lesion area, and the accumulation of foam cells will eventually lead to the formation of a necrotic core in chronic atherosclerosis (117). Macs have an important role in phagocytosis of necrotic cells in the plaques; therefore, pro and anti-inflammatory Macs can help regulate or exacerbate the disease (118). The significant role of the heterogeneous spectrum of pro and anti-inflammatory Macs has been an important area of study over the last decade (119). The polarization of Macs changes their functional phenotype in response to their microenvironment signals. Different polarization of Macs can affect their proliferation and/ or their capability to recruit more monocytes, which will eventually change the abundance and diversity of specific Mac phenotypes in the plaques (120). So far, some different subclasses of Macs have been identified in the plaques based on their surface markers, functions, and cytokine production (Figure 8)(120,121).

M1 Macs in atherosclerotic lesions are stimulated by Cholesterol crystals, Lipopolysaccharide (LPS), Proinflammatory Cytokines, and Oxidized LDL (122). Pro-inflammatory M1 Macs normally get activated through Toll-like receptor (TLR-4) or nuclear factor NF kappa B (NFκB) pathways (123). They secrete pro-inflammatory cytokines such as TNF-a, IL-6, IL-12, IL-1ß, IL-12, and IL-23 (107). M1 Macs are the most abundant Mac phenotype in lipid cores (124). Alternatively, activated M2 Macs produce anti-inflammatory cytokines such as IL-10 and TGF-ß, and they have a high capability of phagocyting dead cells and debris (125).

Also, some subtypes (M2a) have roles in wound healing and angiogenesis (126). So far, some subtypes of M2 Macs have been known in the atherosclerotic lesions: M2a Macs get

activated by IL-4 and IL-13 cytokines (127). M2b subtypes are induced by immune complexes along with IL- ß and LPS, and they specifically express high levels of TGF-b but different from other M2 subtypes they significantly produce IL-1, IL6, and TNF (which are inflammatory cytokines) as well (127, 128). And finally, M2c Macs that get activated by glucocorticoids and TGF- ß (3). Other than M1 and M2 phenotypes, there are a couple of other polarized Macs in the plaques. Ox-phospholipids induce mox Macs, and they express IL1-ß and cyclooxygenase 2 (COX-2) through TLR-2 dependent metabolic pathways. M4 Macs are polarized by CXC chemokine ligand 4 (Cxcl4), and they indicate reduced phagocytosis and produce inflammatory cytokines and molecules such as IL-6, TNF, and MMP-7 (129).

Mac subtypes and their sub-populations can affect the other Macs and eventually modify the microenvironment and induce the plaques' aggravation or regression (120, 129). It is important to note that polarized Macs have the capability to depolarize or switch their phenotypes based on their microenvironment alterations (120).

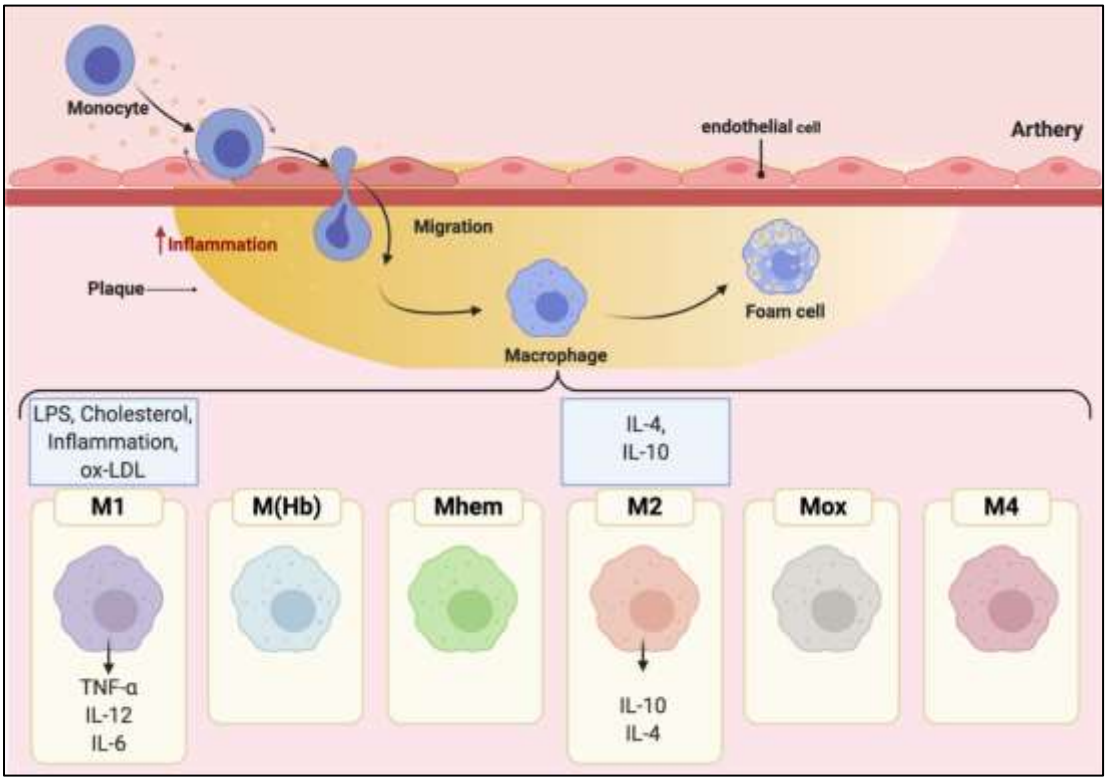


Figure 8 Mac polarization in atherosclerotic plaques

Local inflammatory responses in atherosclerosis activate different cells that are in the atherosclerotic lesion area. The microenvironment of the lesion will differentiate the monocyte to Macs. The Macs will transform into foam cells and, eventually, retain in the plaques.; therefore, pro, and anti-inflammatory Macs can help regulate or exacerbate the disease. The polarization of Macs changes their functional phenotype in response to their microenvironment signals. So far, different subclasses of Macs have been identified in the plaques. M1 Macs in atherosclerotic lesions are stimulated by Cholesterol crystals, Lipopolysaccharide (LPS), Proinflammatory Cytokines, and Oxidized LDL. They secrete pro-inflammatory cytokines such as TNF-a, IL-6, IL-12. M1 Macs are the most abundant Mac phenotype in lipid cores. Alternatively, activated M2 Macs produce anti-inflammatory cytokines such as IL-10 and IL-4. Ox-phospholipids induce Mox Macs to express IL1-ß and cyclooxygenase 2 (COX-2). M4 Macs and produce inflammatory cytokines. M(Hb) and Mhem Macs are in the group of anti-inflammatory subtypes.

Cross talk between innate immunity and lipid metabolism in atherosclerosis

Macs in atherosclerotic lesions are continuously exposed to modified lipids and cholesterols ox-LDLs (130). Cholesterols, LPS, and Ox-LDL and inflammatory cytokines can initiate an inflammatory environment in atherosclerotic lesions and consequently cause activation of M1 inflammatory Macs (131). Cleavage of NLRP3 inflammasome and expression of IL-1ß can be a result of Mac M1 polarization through cholesterol-induced inflammation. In addition, LPS and Ox-LDL can activate Toll-like receptor 4 (TLR-4) signaling and consequently, pro-inflammatory Macs (107). TLR-4 signaling reduces cholesterol efflux through decreasing the expression of the Lipid transporter ATP-binding Cassette Sub-family A member 1 (ABCA1) and ATP Binding Cassette Subfamily G Member 1 (ABCG1) (132). Increased inflammation and inhibited cholesterol efflux in pro-inflammatory Macs will eventually lead to the accumulation of cholesterol and triglycerides in the Macs and increased levels of lipid droplets and foam cell formation (116). TLR2 and specifically TLR4 are the most dominant TLRs in the initiation of atherosclerosis; they can induce signaling pathways to activate MyD88 and NFκ-B and eventually cause secretion of pro-inflammatory cytokines (133). Deficiency of TLR-MyD88 signaling, caused decreased levels of MCP-1 expression, reduced inflammation, and decreased plaque size (134). Also, a study indicated that deficiency of TLR/IL-1R signaling could regulate cholesterol metabolism by reducing inflammation (135). The endogenous molecules or metabolites that can be an agonist or antagonist for TLR2 and TLR4 signaling in atherosclerosis are not fully investigated. Few rodent studies hypothesize that microbiota-derived metabolites can also play an important role in regulating TLR signaling in atherosclerosis (136). Overall, TLR signaling, especially TLR-2 and TLR-4 signaling, are pro-atherogenic signals in atherosclerosis (137).

Lipid metabolism of Macs

The most efficient pathways for producing ATP in the cells is Fatty Acid oxidation or FAO, one molecule of fatty acid (FA), for example, palmitate, can produce 129 ATPs (138,139).

Since Macs are potential cells in phagocytosis, they are very important cells in lipid metabolism as well. Macs can uptake the lipoproteins from apoptotic cells and activate passive and active mechanisms to reduce cholesterol in the cell because excess cholesterol will cause Macs' foam cell formation (140). Through scavenger receptors, phagocytosis, and micropinocytosis, Macs can uptake modified LDLs, and VLDLs and these lipids can get catabolized in the lysosome to free fatty acids (FA) and cholesterols (141). In the ER (Endoplasmic reticulum), the free cholesterols can get esterified again and form cholesterol fatty acid esters, which can accumulate in the cytosol as lipid droplets and shift the Mac towards foam cell formation (142). In an alternative pathway, free cholesterols can get exported through the cell membrane (143). Excessive cholesterol accumulation can lead to increased expression of some transcription factors such as retinoid x receptor (Rxr) and liver x receptor (Lxr) that can upregulate the ABCG1 and ABCA1 expression (144). And these lipid transporters can mediate the efflux of the cholesterol via some intermediate pathways to form HDLs (145). Some other studies suggest the possibility of aqueous diffusion and facilitated diffusion pathway of cholesterol as well (146).

Degradation of the lipids to fatty acids and free cholesterol, in the lysosome, takes place through the acid lipase enzyme, the process that is called lipolysis (147). Cholesterol will get effluxed to LDLs, and the fatty acids will go through the fatty acid oxidation (FAO) pathway (97).

In FAO, through some enzymatic reactions, fatty acids will get activated into fatty acid acyl-CoA and enter the mitochondria through CPT1A (carnitine palmitoyltransferase 1A) enzyme (148). In the mitochondria, carnitine will be removed by CPT-2 enzyme, and β -oxidation of FA-

acyl-CoA starts (148, 149). FAO leads to increased production of acetyl-CoA and through the citric acid cycle, increased production of NADH and FADH that will consequently produce ATP through the electron transport chain in the mitochondria (150).

On the other hand, to activate pathways, proliferate, and grow, Macs need lipids (97). Fatty acid synthesis (FAS) is a pathway that cells can generate FA in the cytoplasm using the metabolites coming from the Krebs cycle, pentose phosphate pathway, and glycolysis (151). Moreover, mTOR activation can induce FAS through a transcription factor named SREBP (sterol regulatory element-binding protein) (152); however, mTOR inhibition can lead to activation of autophagy and Lipophagy (153).

Some studies suggest that FAO activation is one of the main pathways in M2 Macs (138). FAO activation after IL-4 stimulation in M2 Macs is dependent on PPARs and their co-activator PGC1b (Peroxisome proliferator-activated receptor gamma co-activator 1-b) that increases mitochondrial biogenesis (154). The dependency of M2 Macs on FAO is still controversial. Few publications indicated that despite inhibiting FAO in M2 human and mice Macs, etomoxir was not able to reduce M2 polarization in IL-4 polarized M2 Macs (155, 156). Also, in another study in CPT-2 deficient Macs, IL-4 can still induce M2 polarization. But these observations may be explainable by etomoxir dose-dependency justification.

Results regarding M1 Macs are suggesting that when modified LDL and Free FA uptake is increased and when the expression of the scavenger receptor is upregulated, Lipolysis and FAO reduce (156).

In terms of atherosclerosis, the role of different Mac phenotypes and the metabolic pathways (that they use to meet their energy demands and induce their phenotypes) is still not completely unraveled with regards to plaque stability or exacerbation. But overall, M1 Macs

induce more inflammation and have less activated FAO, that is why they accumulate more lipids compared to M2 Macs that have active FAO and Free FA consumption (156).

Modulation of glycolysis in Macs

When Macs get activated with PAMP (via TLR) or some other pro-inflammatory factors, Glycolysis pathways and glucose uptake significantly increases in these M1 Macs and as mentioned before, the OXPHOS pathway decreases (156, 157). Glycolysis is an alternative, faster, but inefficient pathway compared to OXPHOS for ATP production (156). Studies show that inhibition of glycolysis can help in the reduction of M1 polarization of Macs. Reduced OXPHOS activation in pro-inflammatory Macs will result in the accumulation of Krebs cycle metabolites

such as Malate, Fumarate, Citrate, and Succinate (158). These intermediates can be used as precursors for naerobic glycolysis in inflammatory Macs (Figure 9) (159).

M1 Macs have increased aerobic glycolysis, the process of converting glucose into lactate.

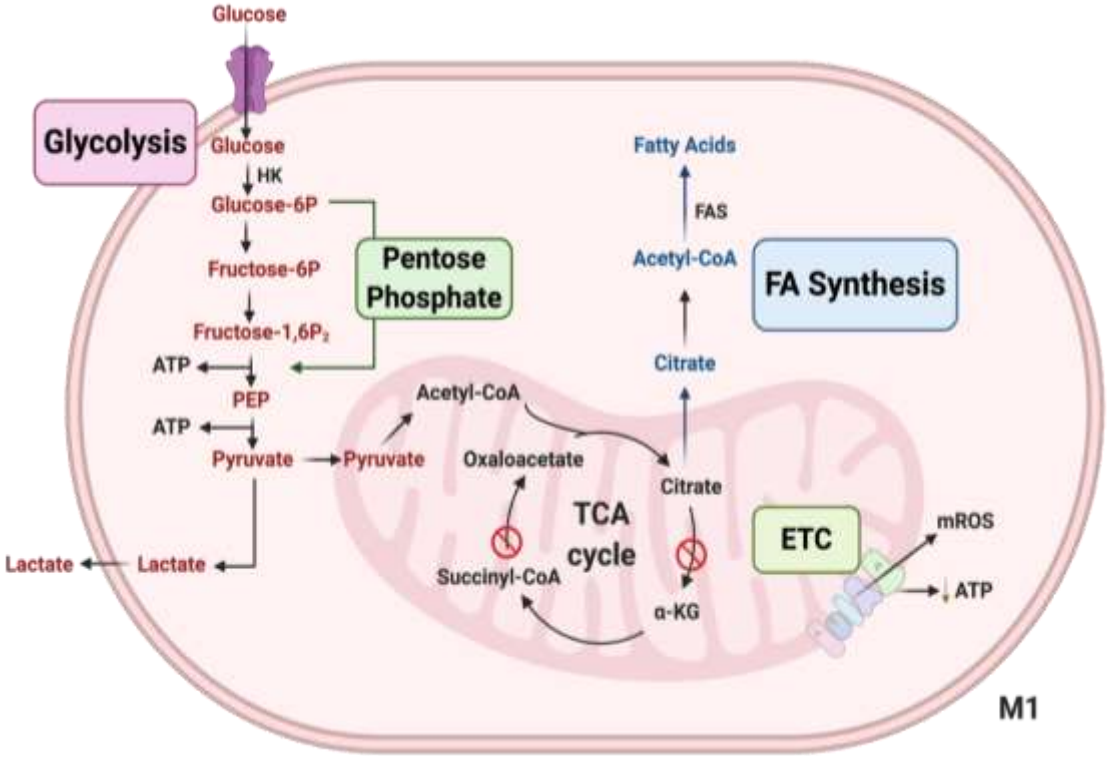


Figure 9. Important metabolic pathways in M1 Macs.

Increased pentose phosphate pathway (PPP) is another characteristic of M1 Macs, which leads to the generation of the inflammatory mediators, nitric oxide (NO) and reactive oxygen species (ROS). M1 Macs have enhanced levels of succinate and citrate because TCA cycle is partially inhibited. Also, increased citrate leads to increased levels of fatty acid synthesis in M1 Macs. Reduced levels of electron transport chain (ETC) activity and production of mitochondrial ROS is another characteristic of M1 Macs.

Furthermore, increased glycolysis will uncouple the mitochondrial electron transport chain from ATP synthesis, causing increased ROS production levels (160). ROS production is one of the mechanisms that Macs use for bacterial killing (161). mTOR, HIF-1a (hypoxia-inducible factor

1-a), and uPFK2 (ubiquitous enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase) are involved in the regulation of glycolysis (162). uPFK2 can increase glycolysis by increasing the PFK-1 (6-phosphofructo-1-kinase) enzyme activity, and consequently, the production of more fructose 2,6-biphosphate in pro-inflammatory Macs (162). Recent studies show that other than LPS, there are other pro-inflammatory factors than can induce the Mac metabolism towards M1 like polarization (163).

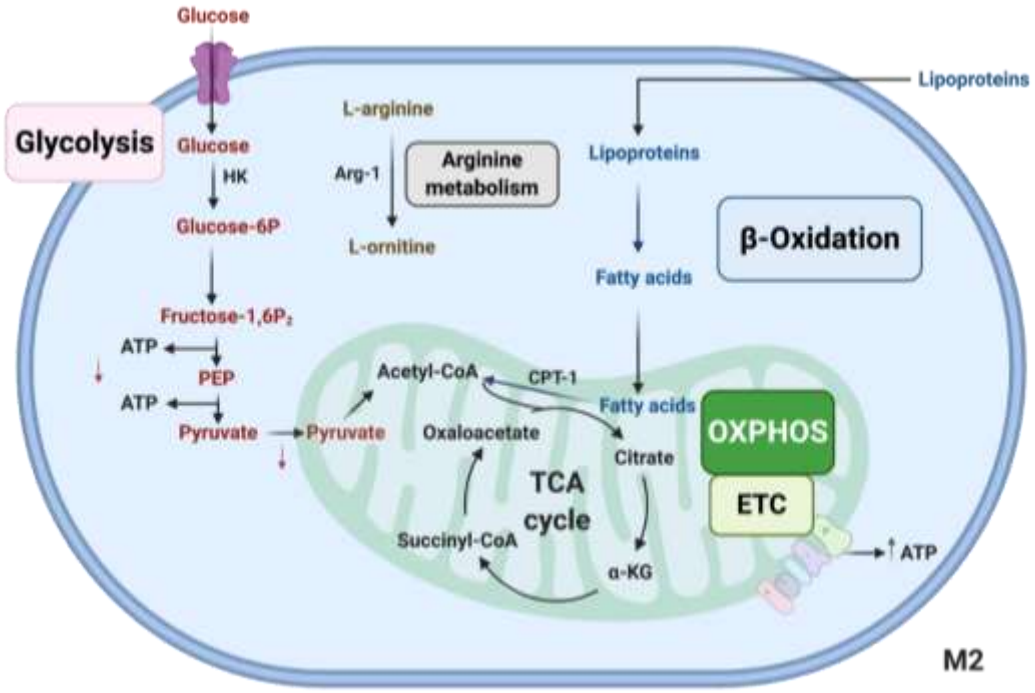


Figure 10. Important metabolic pathways in M2 Macs.

M2 Macs indicate increased ATP production through electron transport chain (ETC) oxidative phosphorylation (OXPHOS). M2 Macs show lower levels of glycolysis and increase levels of fatty acid oxidation (β-Oxidation). Increased arginine metabolism is another characteristic of M2 Macs.

There are also elevated levels of glucose uptake in M2 polarized Macs compared to naïve ones, but in comparison with M1 Macs, the glucose mostly will be used in the Krebs Cycle (164). And as mentioned before, to maintain their anti-inflammatory functions, M2 Macs use the Krebs cycle and OXPHOS pathways. However, some studies suggest that M2 Macs use the FAO pathway to meet their anti-inflammatory energy needs (Figure 10) (165).

Future studies perspective

Future works need to be done to elucidate the mechanisms of actions by which indole induces anti-inflammatory anti-foam cell, pro-autophagic functions. Since indole has substantial beneficial effects in gut and distal organs, cell-type-specific investigations are highly valuable. Although studies have shown that tryptophan metabolism has an important role in the regulation of atherosclerosis (166), the mechanistic role of the indoles through gut microbiota has not been fully unraveled. Other than tryptophan metabolites, different microbiota dependent metabolites such as secondary bile acid metabolism have been reported to have a role in progression atherosclerosis (167). Overall, my study and other studies suggest the importance of microbiota, their dependent metabolites, and the molecular pathways of these metabolites, beyond the basic conventional risk factors of atherosclerosis.

Also, gaining more perspective regarding novel endogenous therapeutics that can regulate Macs metabolism be extremely helpful in atherosclerosis prevention and treatment. Figure 11 is my overall model for this project.

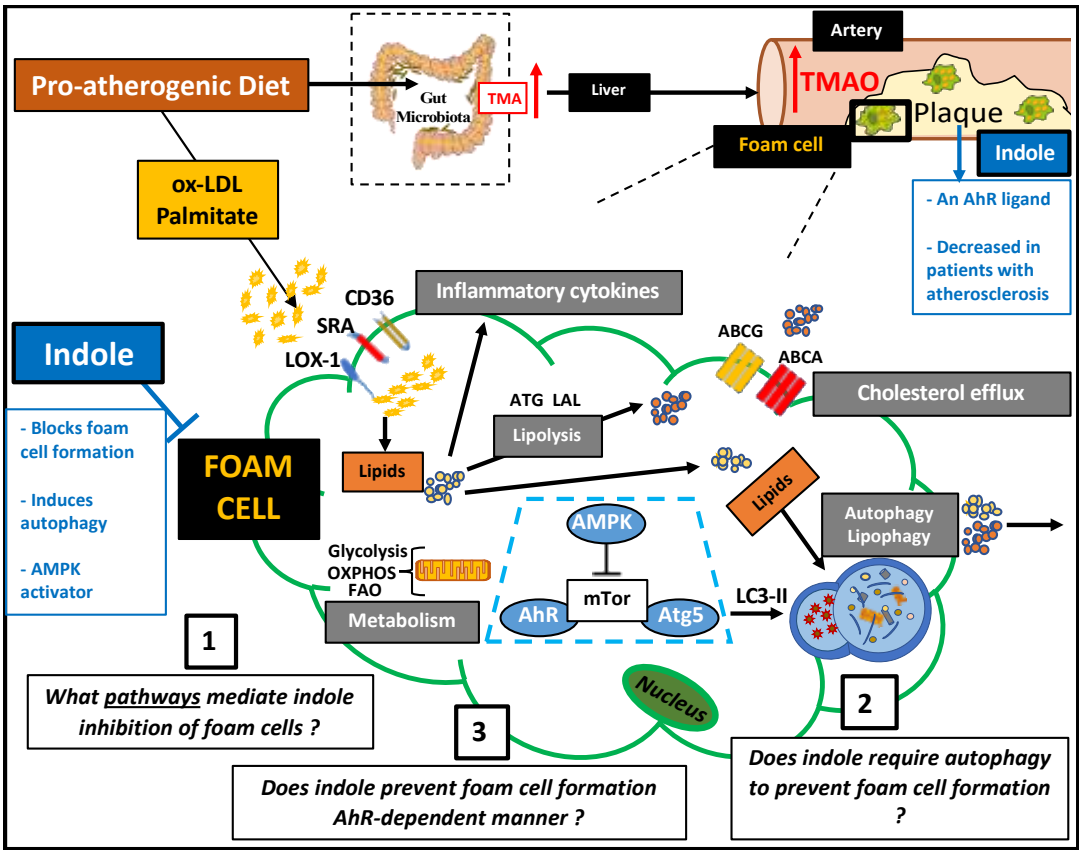


Figure 11. Overall Model.

The microbiota-derived Trp-metabolite Indole augments Mac autophagy and changes the metabolic state of the cells. Besides, Indole inhibits inflammation (based on preliminary data). Our predicted model illustrates that endogenous microbiota-derived Indole and microbiota dependent TMAO enters the circulation and conditions circulating pool of Macs to favor an anti-inflammatory Mac phenotype along with increased basal autophagy. These metabolic conditioned Macs subsequently enter the cardiac vasculature; then, upon proatherogenic conditions, the metabolite conditioned Macs will inhibit atherosclerotic pathology (e.g., plaque formation). Based on our preliminary data AhR, autophagy, and AMPK pathways are involved in Indole's mechanisms of action.

CHAPTER 2

INDOLE LIMITS PHAGOCYTE INFLAMMATION IN RESPONSE TO PRO-ATHEROGENIC LIPOTOXIC STIMULI

Overview

There are very few studies that have reported microbiota metabolites with direct beneficial or detrimental atherogenic functions in reducing or exacerbating atherosclerosis. Macs s are the critical cells in studying atherogenic plaques. There is a wide range of polarized Macs in atherosclerotic plaques. Most of the Mac involved in atherosclerosis are inflammatory or M1 like Mac. Studies have shown that diet, and gut microbiota metabolites directly correlate with positive or negative regulation of atherosclerosis. Indole is a microbiota-derived metabolite abundant in the gastrointestinal tract, and it is also present in the circulation. Both APCs and monocytes present in the GI tract and distal locations, as well as the cells in the circulation, are regularly exposed to this small molecule. We hypothesized that indole is a major microbiota-derived regulator of phagocyte inflammation and metabolism in the GI tract and blood circulation. In this section, my study starts with testing the ability of indole to limit Mac inflammation in response to pro-atherogenic lipotoxic stimuli. My findings in this section are as follow:

1. Indole reduces Mac inflammatory responses to the atherogenic stimuli.
2. Indole reduces the glycolysis and OXPHOS metabolism of Mac.
3. Indole mostly reduced the M1 polarization of Macs and up or down regulates M2 polarization, but it does not shift M1 to M2 phenotypes.
4. Indole regulation of Mac metabolic phenotypes and inflammatory responses are independent of Aryl hydrocarbon receptor (AhR).

Rationale

One of the significant signs of chronic inflammatory disease, atherosclerosis, is the accumulation of pro-inflammatory M1-like Macs in coronary arteries (168). Mechanisms that reduce Mac inflammation are likely to decrease the progression of atherosclerosis. Other than TMAO and SCFA, there have been minimal studies regarding microbiota-derived or dependent metabolites and their relationship with atherosclerosis (169). In our published and unpublished reports, indole is revealed to be a novel microbiota-derived metabolite that is reduced in mice under conditions that promote atherosclerosis (170). Indole is a small molecule that is diffusible from the gut and is present in the circulation (50). When indole is given exogenously to microbial LPS activated phagocytes, it reduces inflammatory cytokines expression. It is reported that Macs in atherosclerosis display a more active metabolism like those observed in inflammatory Macs (171). There is a wide range of polarized Macs in atherosclerotic plaques, and inflammatory or M1 Macs are the most abundant ones (107). There are various inflammatory stimulators to induce an inflammatory phenotype in atherosclerosis-related studies. Lipopolysaccharide (LPS), IFNγ, ox-LDL, TMAO, palmitate, etc. can all induce an inflammatory phenotype in Macs (172). In this study, conventional LPS induced M1 Macs, IL-4 induced M2 Macs, and naïve untreated Macs have been tested for various conditions (172). To check the indole regulation of Mac polarization, I analyzed metabolic parameters such as glycolysis, and Oxidative phosphorylation (OXPHOS) changes in polarized BMDMs by testing extracellular acidification rate (ECAR), and the mitochondrial oxygen consumption rate (OCR), using Seahorse extracellular flux analyzer.

We set out to determine whether indole might promote an anti-atherogenic phenotype and function in Macs. Using AhR deficient mice (Taconic), we determined whether the AhR is the mechanism by which indole exerts its effects on Macs.

Results

Mac and DCs are altered by high-fat diet to produce more inflammatory cytokines

The consumption of high-fat diets and metabolism of the associated saturated fatty acids are important risk factors for developing disorders such as obesity, diabetes, and atherosclerosis (173). The relationship between gut microbiota, energy homeostasis, and inflammation and their role in the pathogenesis of obesity-related conditions is progressively recognized (174). Dendritic cells and Macs are the cellular junctions bridging innate and adaptive immunity (175).

Dendritic cells are a crucial immuno-regulatory cell in the gastrointestinal tract (176), and Macs are the most important immune cells associated with atherosclerosis (107). Mouse models of high-fat diets demonstrate a link between microbiota composition and the development of obesity, diabetes, and atherosclerosis through several mechanisms (177).

We were interested in checking differences in inflammatory responses of Macs and DCs from a high-fat diet and a low-fat diet (Figure 12). And since our previous lab data indicated that indole could reduce splenic APCs inflammatory responses, we set up to investigate Macs and DCs alteration by a high-fat diet in the context of inflammation. The initial steps of culture and indole pretreatment of the samples is illustrated in Figure 13. To perform the experiment, we purchased five, 14 weeks old of each high fat and low-fat diet C57BL/6J mice (Figure 12). Then we stained spleen Macs, and DCs with/without LPS and checked the expression of TNF-a (Figure 14) and IL-12 (Figure 15.). our data indicated that Mac and DCs altered by high-fat diet to produce more TNF-α and IL-12. Suggesting that high fat diet even without stimulation with LPS can induce inflammation in Macs and DCs.

A

	Control Diet D12450B (10 kcal% fat, 3.8 kcal/gram)		High Fat Diet D12492 (60 kcal% fat, 5.2 kcal/gram)	
	Grams %	kcal %	Grams%	kcal %
Protein	19	20	26	20
Carbohydrate	67	70	26	20
Fat	4	10	35	60
Total		100		100

B

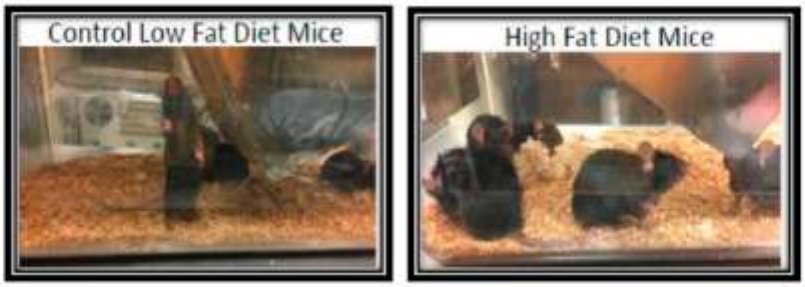


Figure 12. High Fat and control diet mice used for the detection of inflammatory macs and DCs.

(A) Diet Information of C57BL/6J DIO mice that were maintained on D12492(60 kcal% fat) and control mice that were kept on D12450B (10 kcal% fat) (B) Picture of high-fat diet and low-fat diet mice used for investigating splenic Mac and DC inflammatory states.

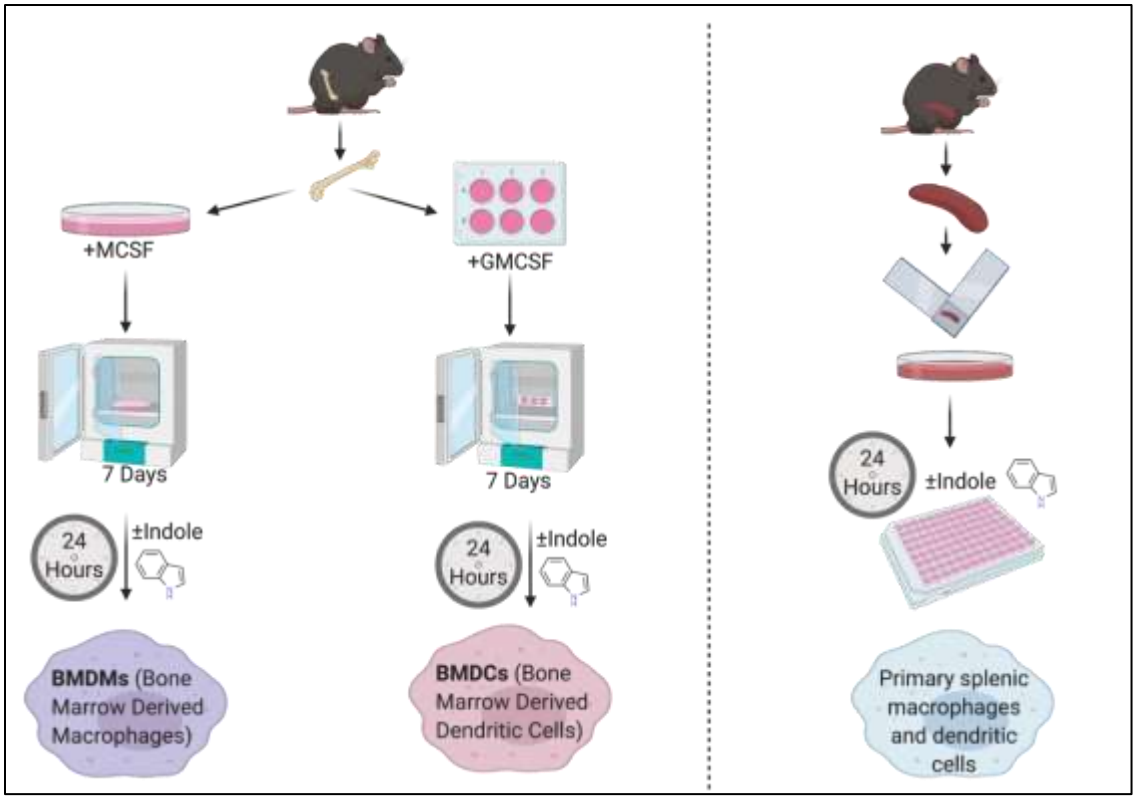


Figure 13. Culture method and Indole pretreatment of bone marrow-derived and primary splenic Macs and dendritic cells.

(A) To make Bone Marrow-Derived Macs (BMDMs), Bone marrow-derived cells from the femur of 57BL/6 mice were exposed to 10ng/ml MCSF for seven days (left) and media was replenished on day 3. For Bone Marrow-Derived Dendritic Cells (BMDCs), bone marrow-derived cells from femur were exposed to 20ng/ml GMCSF for seven days (right) media was replenished on day 2 and 5. Mature BMDMs and BMDCs then incubated for another day with indole for further experiments. (B) 57BL/6 mice were smashed between two frosted microscopic slides. Single-cell suspension plated in a 96 well plate in the presence of indole or control (DMF). By using surface markers, Macrophages, and dendritic cells identified through FACS.

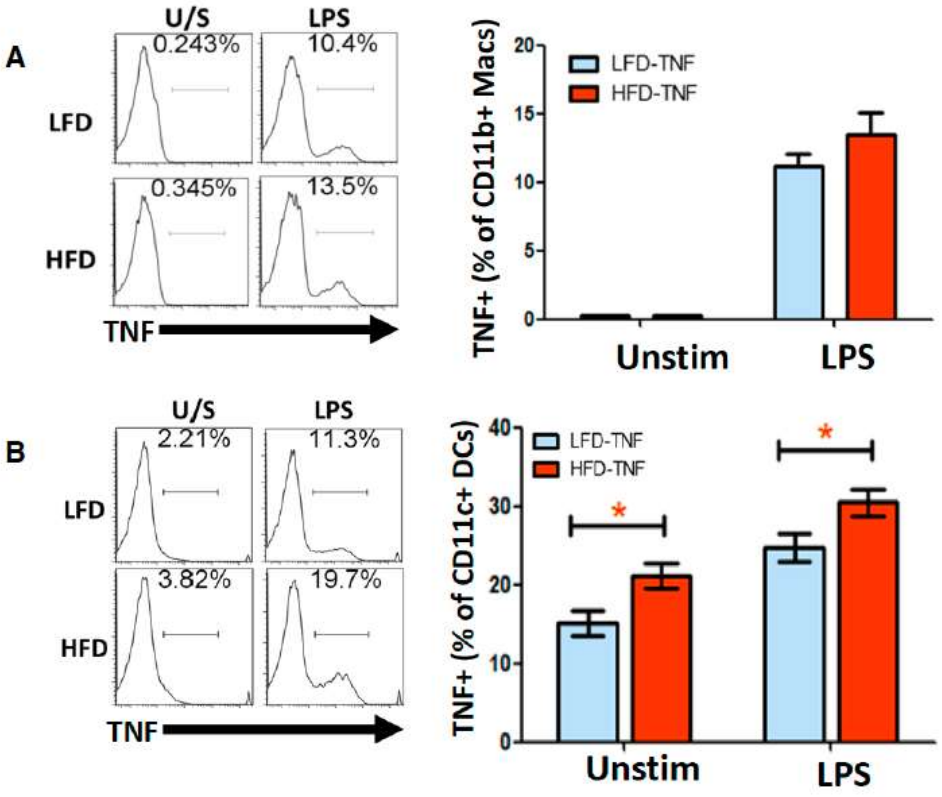


Figure 14. Mac and DCs altered by high-fat diet to produce more TNF-α.

Spleen cells ($1x10^6$) form HFD/LFD were stimulated or not with LPS (1µg/ml) in a 96 well U-bottom tissue culture plates in the presence of Golgi plug (1:1000 final) for 4 hr. After, cells were stained with (A) anti CD11b and (B) anti CD11c to identify Macs and DCs. Then cells were stained intracellularly for TNF-α.

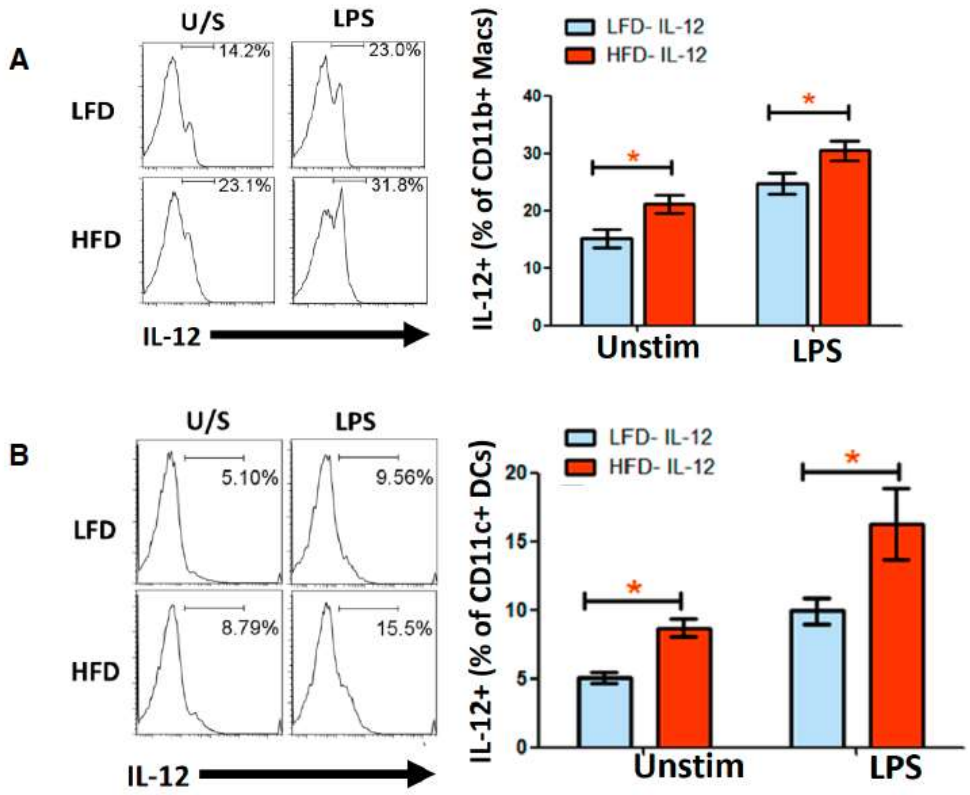


Figure 15. Mac and DCs altered by high-fat diet to produce more IL-12.

Spleen cells ($1x10^6$) form HFD/LFD were stimulated or not with LPS (1µg/ml) in a 96 well U-bottom tissue culture plates in the presence of Golgi plug (1:1000 final) for 4 hr. After, cells were stained with (A) anti CD11b and (B) anti CD11c to identify Macs and DCs. Then cells were stained intracellularly for IL-12.

Indole reduces TNF production of inflammatory BMDMs and BMDCs

In order to test pro-inflammatory cytokine production in Macs and DCs., we generated 95% pure bone marrow-derived Macs (BMDMs) using CD11b and F4/80 surface detection markers and dendritic cells (BMDCs) from the high fat and low-fat diet mice (Culture steps are illustrated in Figure 13).

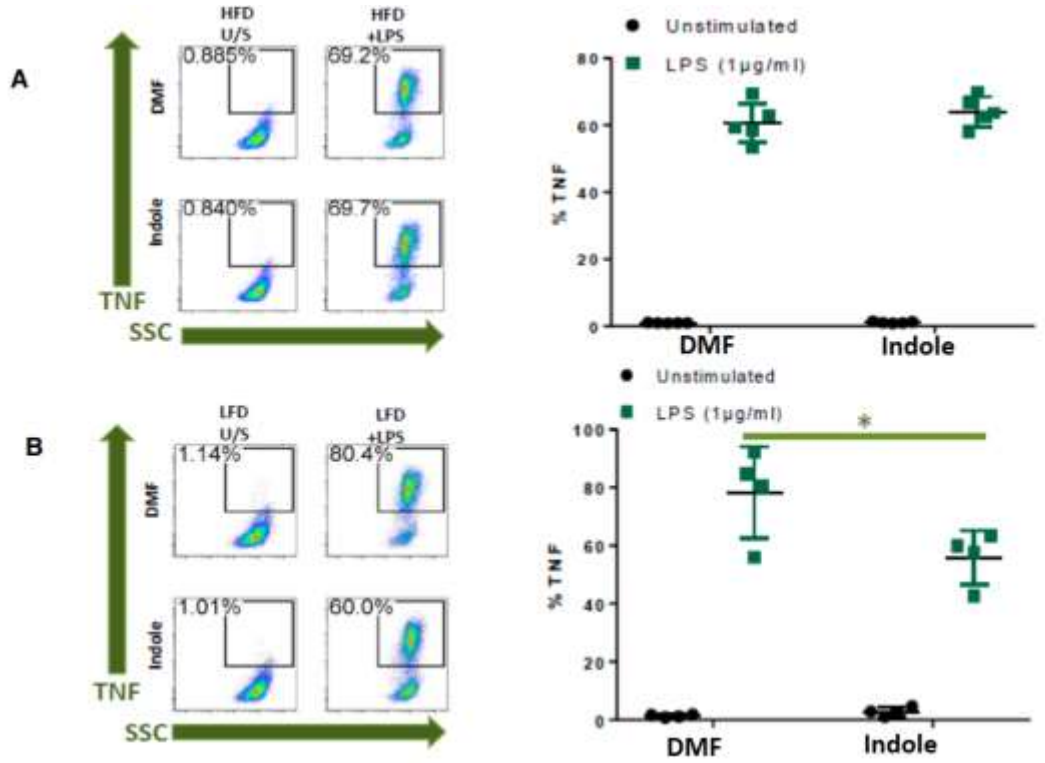


Figure 16. Indole reduces TNF in BMDMs from low fat diet mice but not high fat diet mice.

Indole pretreated (2x 105) bone marrow derived Macs (BMDMs) from high fat diet (HFD) (A) and low-fat diet (LFD) (B) stimulated with DMF or LPS (1µg/ml) in 96 well U-bottom tissue culture plates in the presence of Golgi plug (1:1000 final) for 4 hr. Then cells were stained intracellularly for TNF.

We pretreated day 7 BMDMs and BMDC with indole for 24 hours and stimulated them with LPS for 4 hours. Indole pretreatment resulted in a reduction of TNF production on BMDMs from a low-fat diet but did not change the Inflammatory state of BMDMs from a high-fat diet (Figure 16). Perhaps high fat diet and LPS induction together activated other pathways in the cell, and this caused resistance of these BMDMs to indole's effects. However, indole reduced the TNF production both in high-fat-diet and low-fat-diet LPS-induced BMDCs (Figure 17). Overall results from previous lab experiments (data not shown) and my experiments show that indole can reduce

most of the inflammatory cytokines in Macs and DCs. Data suggest the anti-inflammatory role of indole in APCs.

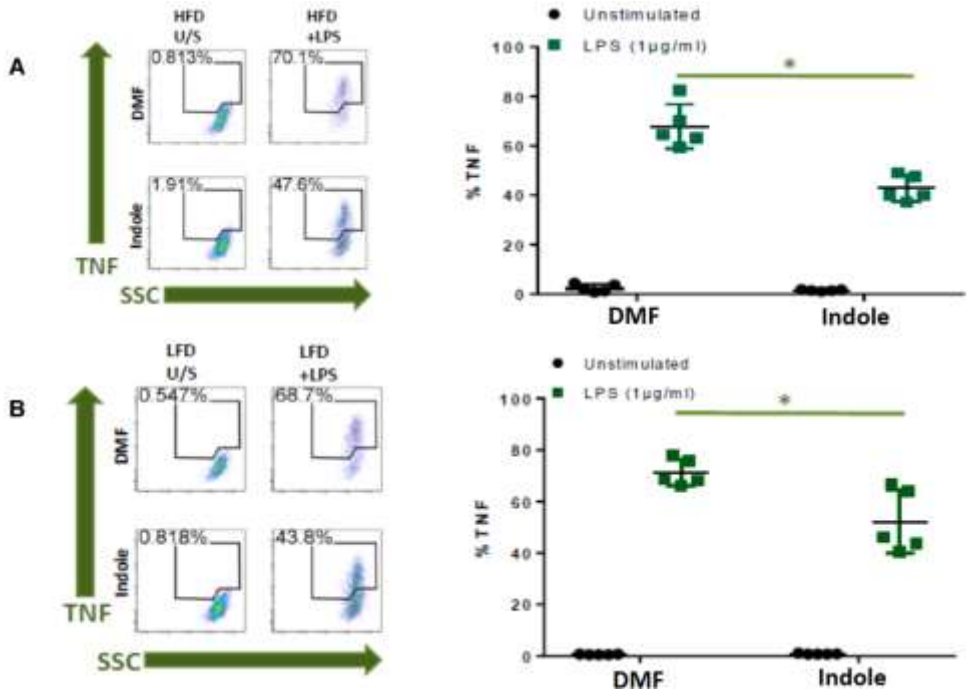


Figure 17. Indole reduces TNF in BMDCs from both high and low-fat diet mice.

Indole pretreated (2x 105) bone marrow-derived dendritic cells (BMDCs) from high-fat diet (HFD) (A) and low-fat diet (LFD) (B) stimulated with DMF or LPS (1µg/ml) in 96 well U-bottom tissue culture plates in the presence of Golgi plug (1:1000 final) for 4 hr. Then cells were stained intracellularly for TNF.

Studies have indicated that saturated fatty acids from high-fat diets can stimulate inflammation in tissues (especially adipose tissue) (178). Atherosclerosis is associated with diets that contain high levels of saturated fatty acids (179). This association between CVD, obesity, inflammation, and the microbiota has led us to hypothesize that microbiota-derived metabolites in the GI tract might regulate dietary saturated fatty acid (Palmitate) induced inflammation. To test this hypothesis, we

generated BMDMs and BMDCs. We examined the effects of a microbiota-derived metabolite, indole, on these cells in the context of saturated fatty acid-induced inflammation.

We investigated pro-inflammatory cytokine production and extracellular metabolic flux. We have previously reported that indole is abundant in the healthy mammalian gut and positively influences intestinal health (180). Also, our previous lab data has shown that indole has inhibitory effects on inflammatory bone marrow-derived Macs and DCs. Here, we demonstrate that indole can reduce saturated fatty acid-derived inflammation in BMDCs and BMDMs (Figure 18.).

Indole pretreated (2x 10^5) bone marrow-derived Macs (A) and dendritic cells (B) stimulated with BSA or palmitate (300µM) in 96 well U-bottom tissue culture plates in the presence of Golgi plug

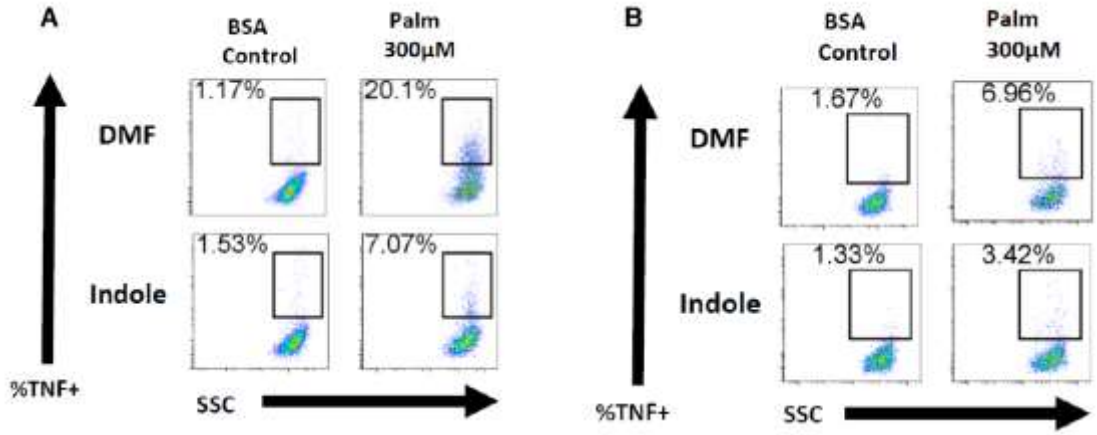


Figure 18. Indole pretreatment reduces palmitate stimulated TNF production.

(1:1000 final) for 4 hr. Then cells were stained intracellularly for TNF.

Indole does not alter APC maturation

Normally APCs, especially Macs and DCs, indicate higher levels of maturation markers after their exposure to a stimulus or phagocytosis (181). In order to make sure that the effects of indole were not due to alterations in the maturation markers, we cultured BMDCs with the presence of indole on day 0, 3, and 6 in their culture media. Then we stimulated them with LPS overnight. Then, BMDCs were stained for CD86. Data suggested that indole pretreatment does not change the maturation of BMDCs in comparison with the DMF solvent control (Figure 19). Both LPS stimulated BMDCs from DMF and Indole groups, reveal the maturation patterns even after 1,4 and 7 days of indole exposure. This suggests that indoles effects are not due to alterations in the maturation factors of APCs.

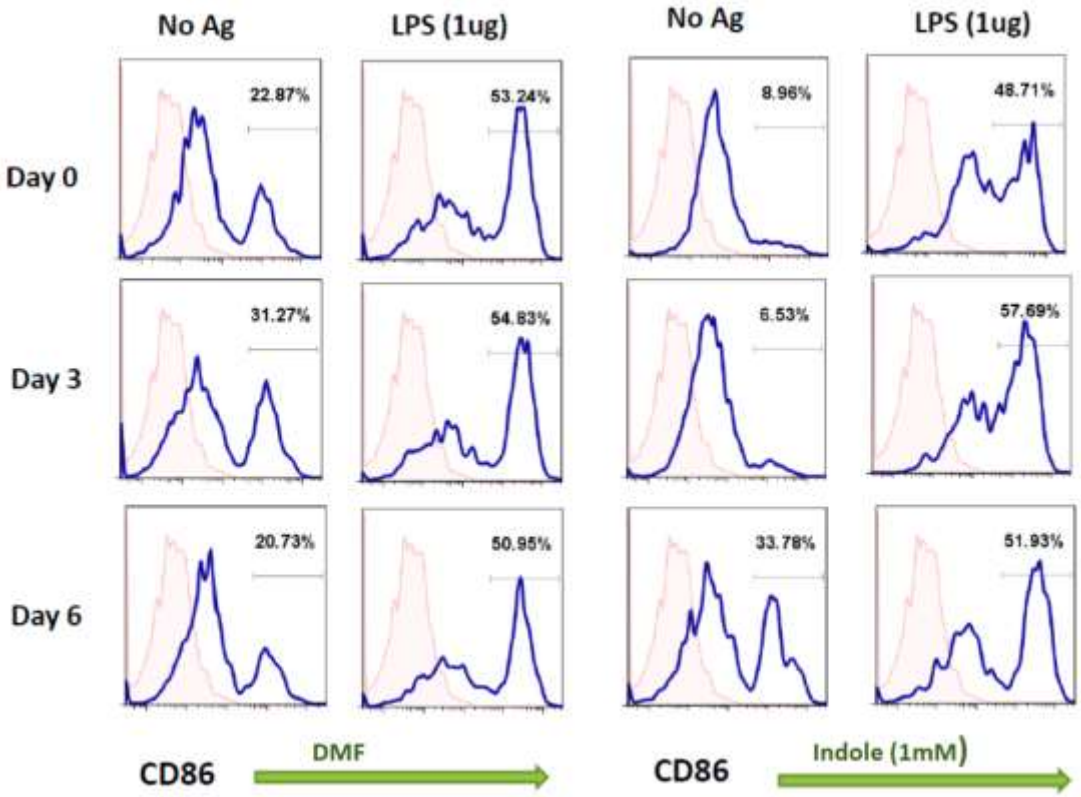


Figure 19. Indole does not alter APC maturation.

Day 0, Day 3, and day 6 Indole/DMF pretreated (2x 105) bone marrow-derived dendritic cells (BMDCs) stimulated overnight (24h) with LPS (1µg/ml) or not in a 96 well U-bottom tissue culture plates. Then cells were stained for CD86. In representative histograms, blue lines indicate indole/DMF-treated DCs, and shaded pink areas are unstained controls.

Indole Alters Mac polarization, favoring towards reduction of M1 polarization, and the activity of indole is AhR independent

CD38

As mentioned earlier in the previous chapter, Macs polarize in atherosclerotic plaques (116). There is a variety of polarized Mac, especially M1 Macs are most of the Mac phenotype in atherosclerotic plaques (123,125). The spectrum of Macs in atherosclerosis is shown in the

introduction section (Figure 8) (182). The physiology and pathology of the M1 Macs have an important effect on monocyte recruitment to the lesion area, plaque initiation, development, progression, vulnerability, and size (182). Also, the M1 phenotype is involved in the maintenance of the inflammatory state in the plaque and consequently increased levels of Mac to foam cell transformation (182). However, it is important to mention that M1 Macs are not the only inflammatory polarized Macs in the plaques (). And the ratio of different polarized Macs in the plaques is a very important factor in the stability and vulnerability of the plaques (112,116). M1 is the most known inflammatory Mac in the plaques and there are detection markers to distinguish LPS or LPS+IFN γ induced M1 Macs (183).

CD38 is an important M1 marker. The expression of CD 38 in LPS induced M1 BMDMs is always higher than M0 and M2, and this makes it a good detection marker (183, 184). CD38 is one of the major cellular NADases in humans and mice. NAD has roles in important physiological processes such as energy metabolism, cardiac and neural processes, aging, senescence, and insulin secretion (184).

In order to test the role of indole in the polarization of Macs, we pretreated the day 7 BMDM with indole (1mM) and then polarized them with polarization skews for 24 hours.

M1 Macs were exposed to 10ng/ml LPS, M2 polarized with 20ng/ml of IL-4, and M0 or naïve Macs just incubated with media. Cells were stained for CD11b and F4/80 to detect Macs and CD38 for the detection of M1 Macs. Mean Fluorescent Intensity (MFI) of CD38 was measured in M1 BMDMs. Data were analyzed with FACS. CD38 gene expression was performed using qRT-PCR.

As expected, the results indicated an increased expression of CD38 in M1 Macs compared to M0 and M2. Indole pretreatment reduced more than 50% of CD38 expression in the M1

phenotype (Figure 20). Data is suggesting that indole is activating mechanisms to prevent M1 polarization.

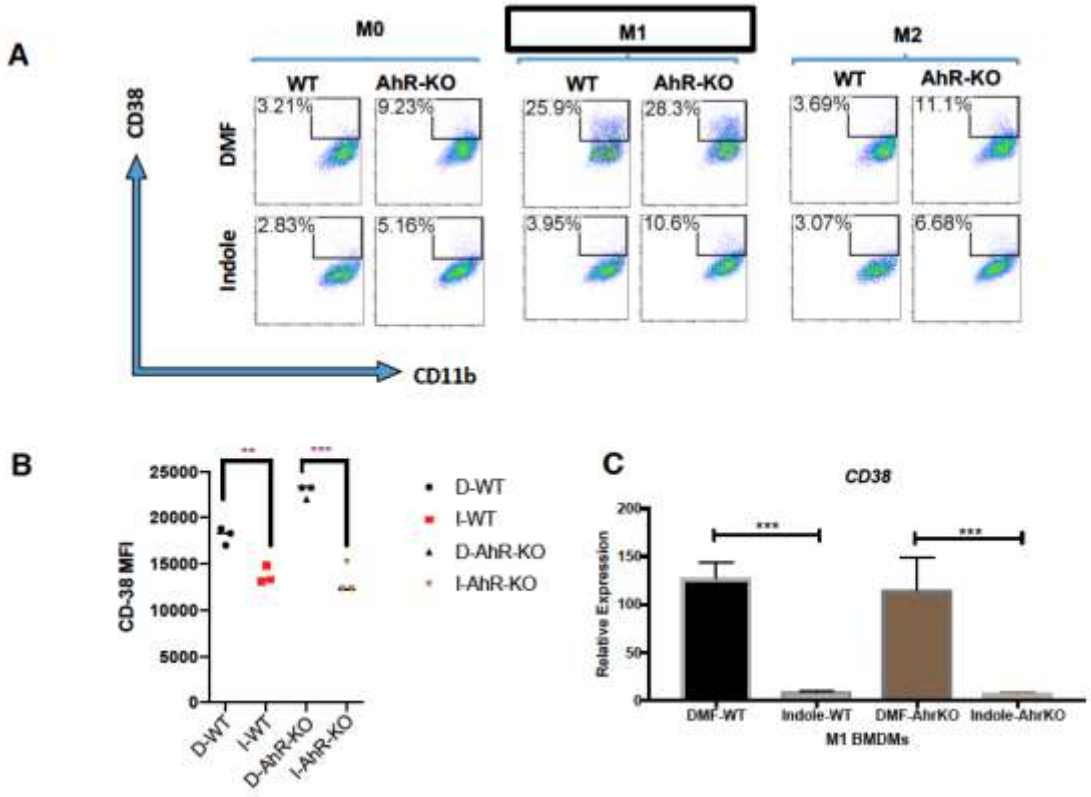


Figure 20. The indole-mediated inhibition of the M1 marker, CD38, in BMDMs is AhR independent.

Day 7 BMDMs were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) M2 (20ng/ml IL-4) (A) Flow cytometric analysis of BMDMs from WT or AhR-KO mice, (2x 105) cells stained extracellularly for CD11b and F4/80 as Mac markers and CD38 as M1 marker using FACS. The percentage of CD38-positive cells are indicated on plots. (B) mean fluorescence intensities (MFI) of the CD38-positive population. (C) Relative gene expression of M1 marker, CD38, measured using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Indole increases the expression of iNOS in M1 BMDMs, and the activity of indole is AhR independent

iNOS

Nitric oxide (NO) is essential in vascular function, immune regulation, and host defense mechanisms. NO is an essential inflammation mediator in the immune system (185, 186). NO is produced in many types of cells and is synthesized with nitric oxide synthase (NOS) (187).

inducible nitric oxide synthase is (iNOS) is one of the three isoforms of NOS (185-187). iNOS can be produced in the cell after the stimulation of pro-inflammatory cytokines (185-187). NO has two important functions with regard to the immune system. First, it has a microbe killing function and can protect the body against microbial and viral infections (188). Second, high production of NO in the cell and tissue can damage the cell or induce them towards an inflammatory state (189). But generally, the effects of NO in monocytes and Macs are involved in anti-microbial protection (190). Furthermore, published data suggest that iNOS in Macs and DCs have roles in their differentiation and regulation (188). NO regulates the cells by nitrating some molecules, transcription factors of the key pathways (191). In Macs, iNOS is listed as an M1 marker (186). Although our previous lab data has shown that indole has anti-microbial killing (data has not shown), and my data indicated that indole reduces CD38, an M1 marker expression (Figure 21), we became interested in checking role of indole in the regulation of iNOS.

We pretreated day 7 BMDMs from WT and AhR-KO mouse with indole or DMF solvent control overnight then stimulated the cells with polarization skews for 24 hours. M0 (Media), M1 or (10ng/ml LPS), and M2 (20ng/ml IL-4). Cells stained extracellularly for CD11b and F4/80 as Mac markers and intracellular staining used for detection of iNOS using FACS. The percentage of iNOS-positive cells are indicated on plots. mean fluorescence intensities (MFI) of iNOS-positive

M1 BMDMs population is measured (Figure 21. B.). Relative gene expression of iNOS, measured in M1 BMDMs using qRT-PCR.

Our results indicate a slight increase of iNOS in gene level (Figure 21. C) and protein level (Figure 21. A). Although NO production has been suggested as an M1marker indicator. Data about iNOS and its effects on polarization of m Macs is controversial. Some data suggest that No production derived from iNOS can reduce inflammasome activated IL-1β production (192). However, we think that increase in iNOS production via indole can be due to the anti-microbial characteristics of indole. Furthermore, the activity of indole in increasing iNOS expression in M1 Macs is AhR independent suggesting that indole is activation other pathways to regulate these Macs.

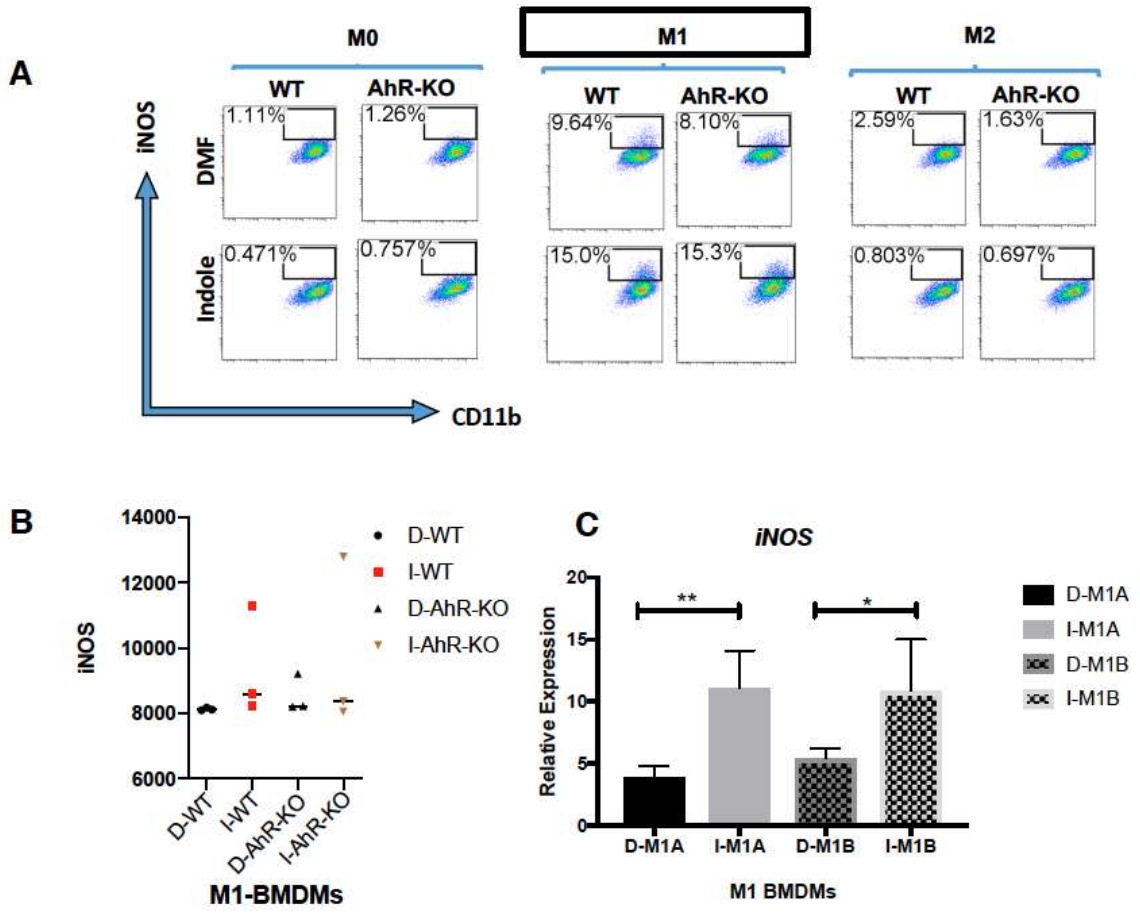


Figure 21. Indole increases the expression of <u>iNOS</u> in M1 BMDMs, and the activity of indole is AhR independent.

Day 7 BMDMs were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 or M1A (10ng/ml LPS), and M2 (20ng/ml IL-4) (A) Flow cytometric analysis of BMDMs from WT or AhR-KO mice. (2x 105) cells stained extracellularly for CD11b and F4/80 as Mac markers and intracellular iNOS using FACS. The percentage of iNOS-positive cells are indicated on plots. (B) mean fluorescence intensities (MFI) of iNOS-positive population. (C) Relative gene expression of iNOS, measured in M1 BMDMs using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Indole decreases the expression of MCP-1 in M1 BMDMs, and the activity of indole is AhR independent

MCP-1

Monocyte chemoattractant protein-1 (MCP-1) or C-C Motif Chemokine Ligand 2 (CCL2) is a chemokine that has a role in the recruiting and infiltration of the monocytes and Macs. MCP-1 has an essential role in the migration of monocytes/ Macs into the arterial wall's endothelial cells (193). MCP-1 inhibitors have been used to prevent atherosclerotic lesion formation (194). Drugs that inhibit MCP-1 have been useful in the reduction of the development of atherosclerosis (194). Gene expression of MCP-1 has been found in the vascular muscle cells, endothelial cells, and Macs of atherosclerotic patients (195). MCP-1 is also an M1 marker (196). That's why our expectation was that indole could reduce the MCP- gene expression. In order to test this, we used day seven WT and AhR-KO BMDMs and pretreated them with indole or DMF control overnight then stimulated them with M1 polarization skew (10ng/ml LPS) for 24 hours. Relative gene expression of M1 marker, MCP-1, measured using qRT-PCR. Data indicated that indole downregulated MCP-1 gene expression in Macs (Figure 22). Data suggests that indole can be a good candidate to reduce the migration of the Macs across the vascular endothelium and reduce the M1 polarization of the Macs.

The effects of indole in the reduction of MCP-1 expression in M1 BMDMs were AhR independent. Data is Suggesting that indole is using other pathways than AhR to downregulate the expression of MCP-1.

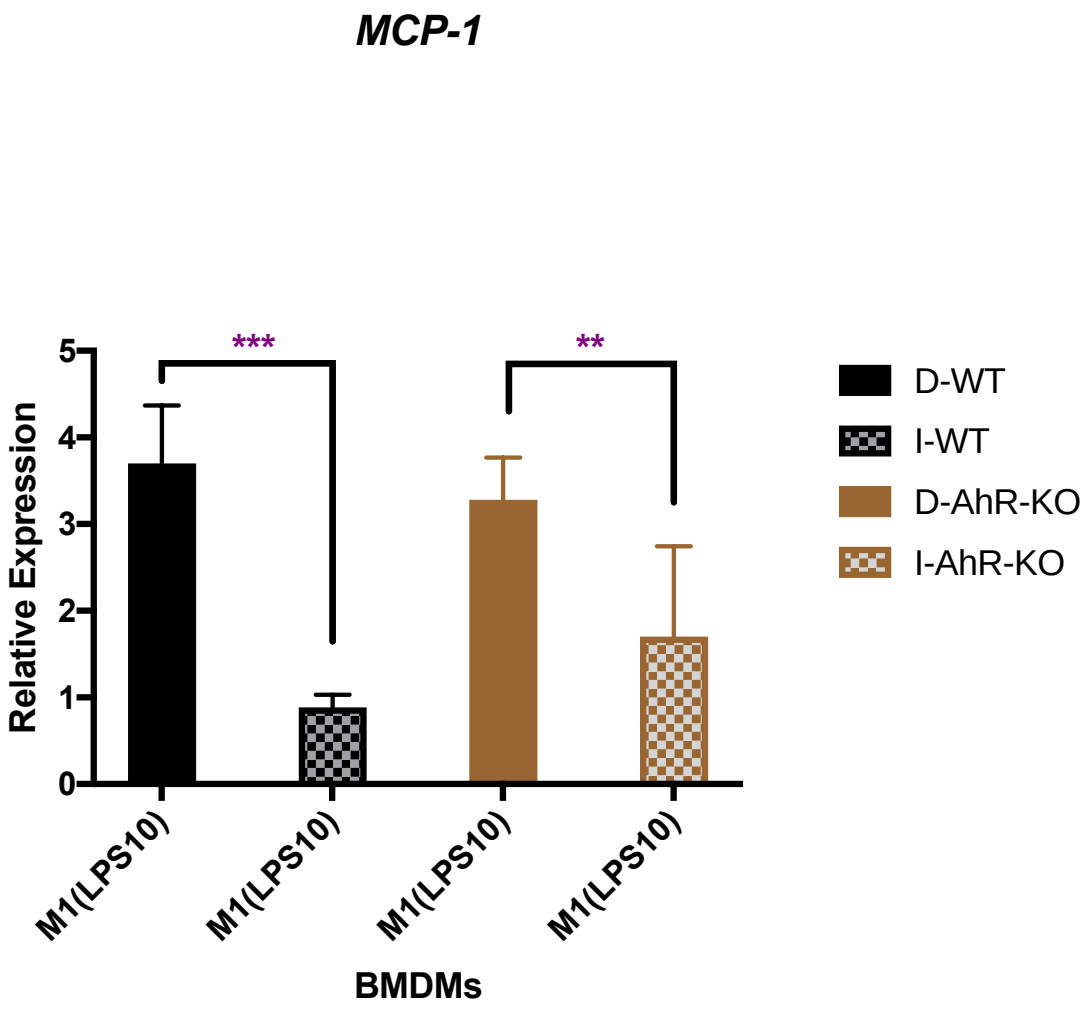


Figure 22. Indole decreases the expression of MCP-1 in M1 BMDMs, and the activity of indole is AhR independent.

Day 7 BMDMs from WT and AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with M1 polarization skew (10ng/ml LPS) for 24 hours. Relative gene expression of M1 marker, MCP-1, measured using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Indole decreases the expression of CD206 in M2 BMDMs, and the activity of indole is AhR independent

CD206

CD206 or MRC1 (C-type mannose receptor 1) is an M2 Mac cell surface protein in mice and humans (197). CD 206 is a transmembrane glycoprotein. CD206 is expressed in resident Macs of various tissues such as cardiac, adipose, and peritoneal tissues (198). Macs that express high levels of CD206 have anti-inflammatory roles, as mentioned in chapter 1 regarding M2 Macs. In atherosclerotic plaques, CD 206+ Macs may increase plaque stability. In order to check the role of indole on the expression of CD206 in the polarization of M2 Macs, we used day 7 BMDMs from WT and AhR-KO mice and pretreated the cells with indole or DMF solvent control overnight. Then we stimulated the cells with polarization skews for 24 hours: M0 (Media), M1 (10ng/ml LPS), and M2 (20ng/ml IL-4). (2x 10^5) cells stained extracellularly for CD11b and F4/80 as Mac markers and CD206 as M2 marker and analyzed using FACS. The percentage of CD206-positive cells are indicated on plots. (Figure 23. A). Mean fluorescence intensities (MFI) of the CD206-positive M2 population were measured (Figure 23. B). Relative gene expression of M2 marker, CD206, measured using qRT-PCR (Figure 23. C). Our data indicated that indole is reducing the CD206 expression in M2 BMDMs. The role of indole in the reduction of CD206 expression is independent of AhR. Since generally, indole has anti-inflammatory properties, we expected that indole would increase the CD206 marker. But these data suggest that indole is not completely shifting the M1 polarization towards M2 polarization, and it is inducing a new phenotype. To better understand the role of indole on the polarization of Macs, we measured other M2 markers in indole pretreated, polarized BMDMs, and we have noticed that indole has both increased and decreased

some M2 markers. This is adding more support to the hypothesis that indole is inducing a new Mac phenotype.

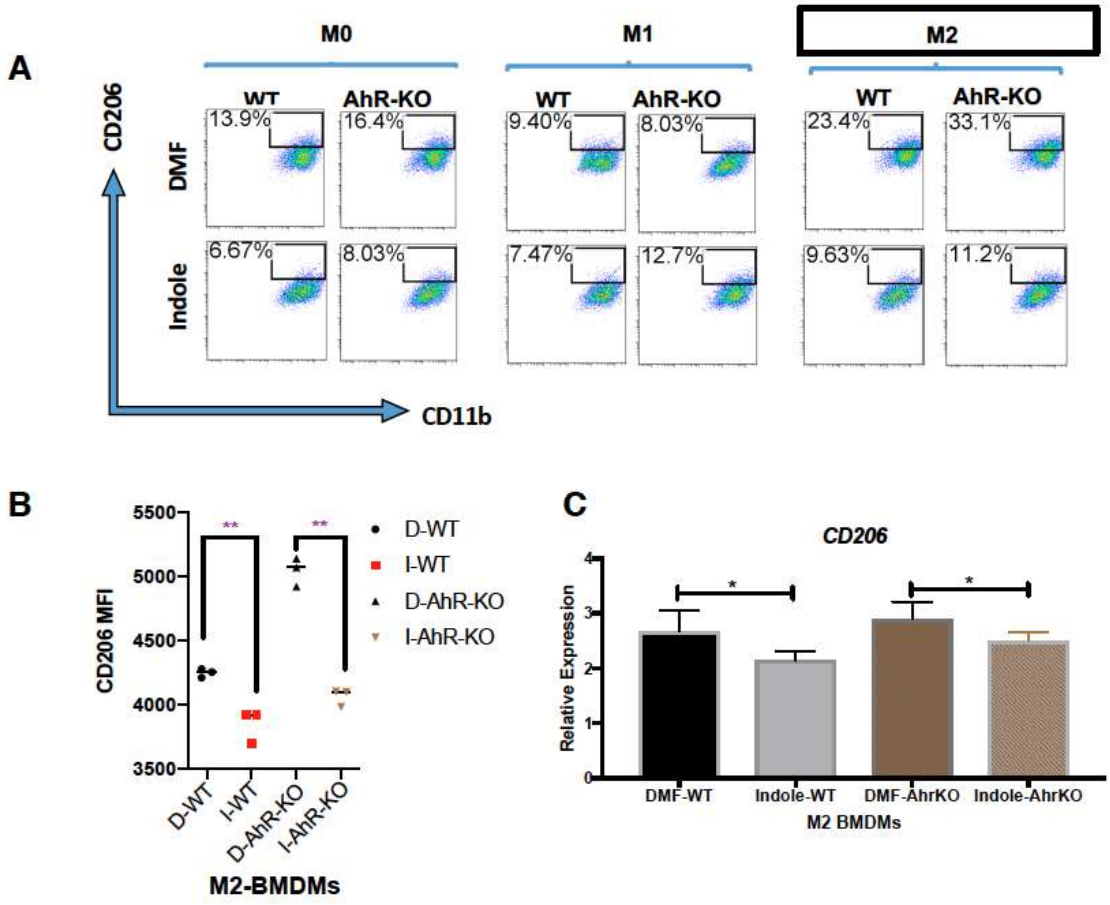


Figure 23. Indole decreases the expression of CD206 in M2 BMDMs, and the activity of indole is AhR independent.

Day 7 BMDMs were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) and M2 (20ng/ml IL-4) (A) Flow cytometric analysis of BMDMs from WT or AhR-KO mice, (2x 10^5) cells stained extracellularly for CD11b and F4/80 as Mac markers and CD206 as M2 marker using FACS. The percentage of CD206-positive cells are indicated on plots. (B) mean fluorescence intensities (MFI) of CD206-positive population. (C) Relative gene expression of M2 marker, CD206, measured using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between

indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Indole decreases the expression of Arg-1 in M2 BMDMs, and the activity of indole is AhR independent

Arg-1

Arginase-1(Arg-1) is another phenotype for M2 Macs. However, Arg-1 is expressed in all types of Macs, but its expression is more in M2 Macs (199). Arg-1 is an enzyme that converts L-arginine amino acid to L-ornithine (Figure 10) and Urea. L-ornithine can then enter the collagen biosynthesis pathway and help in tissue healing (200). TGF-β family can induce tissue healing properties of arginase-1. Some data show that the usage of L-arginine as a substrate for Arg-1 can inhibit L-arginine related immune functions (201). Arg-1 gene in the mouse is amplified by IL-4, IL-13, and TGF-β (200). In order to test the role of indole in M2 polarization of the BMDMs and the dependency of the indole's role on AhR, we used Day 7 BMDMs and pretreated the with indole or DMF solvent control overnight. Then we stimulated the BMDMs with polarization skews for 24 hours as follows: M0 (Media), M1 (10ng/ml LPS), and M2 (20ng/ml IL-4). Then we analyzed the BMDMs from WT or AhR-KO mice. ($2x\ 10^5$) cells stained extracellularly for CD11b and F4/80 as Mac markers and Arg-1 as M2 marker using FACS. Percentage of Arg-1-positive cells is indicated on plots (Figure 24. A). mean fluorescence intensities (MFI) of the Arg-1-positive M2 population were measured (Figure 24. B). Also, the relative gene expression of the M2 marker, Arg-1, was measured using RT-qPCR (Figure 24. C). our results show that indole reduced the expression of Arg-1 in all types of Macs, especially in M2 BMDMs, both in protein level (Figure 24. A, B) and gene-level (Figure 24. C) independent of AhR. So far, other than CD206, Arg-1 is

another M2 marker that indole reduces its expression. Our data is suggesting that indole is not switching the M1 polarization to M2 polarization.

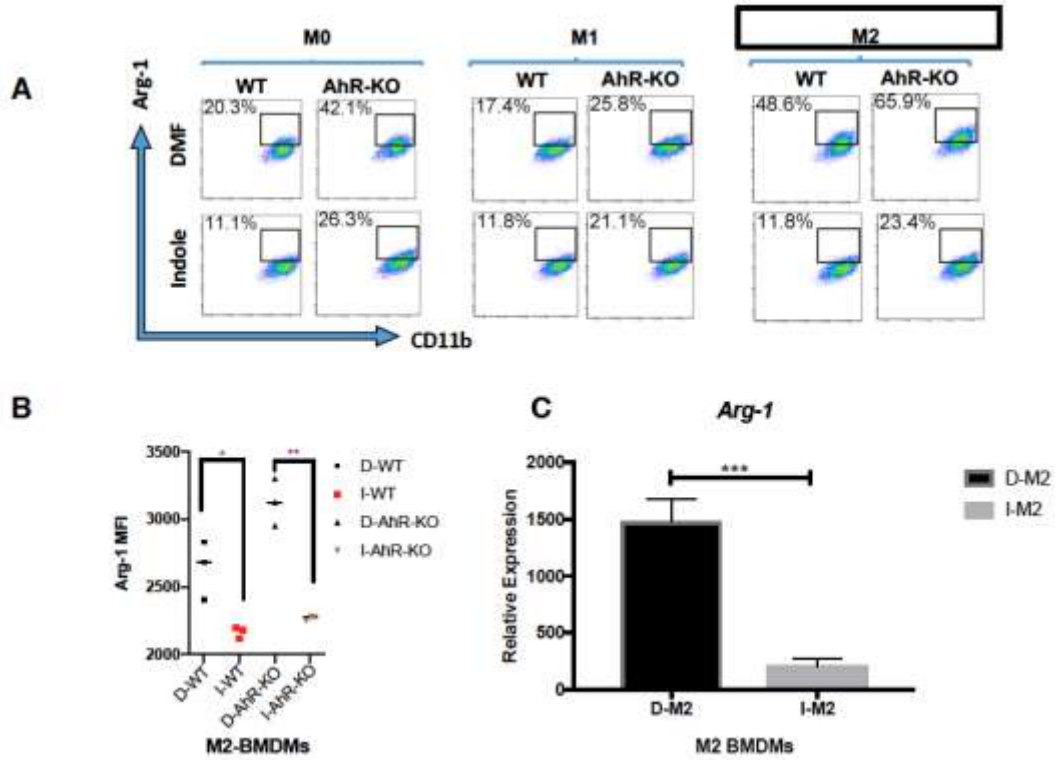


Figure 24 Indole decreases the expression of Arg-1 in M2 BMDMs, and the activity of indole is AhR independent.

Day 7 BMDMs were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS), and M2 (20ng/ml IL-4) (A) Flow cytometric analysis of BMDMs from WT or AhR-KO mice. (2x 10^5) cells stained extracellularly for CD11b and F4/80 as Mac markers and Arg-1 as M2 marker using FACS. Percentage of Arg-1-positive cells are indicated on plots. (B) mean fluorescence intensities (MFI) of the Arg-1-positive population. (C) Relative gene expression of M2 marker, Arg-1, measured using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Indole decreases the expression of Egr-2 in M2 BMDMs, and the activity of indole is AhR independent

Egr-2

Egr-2 (Early Growth Response 2), or Krox20, is a transcription factor that has been proposed as an M2 marker in Macs (183). Egr-2 is essential for the expression of the PPARγ transcription factor (another M2 marker) in Macs (202). A study shows that the siRNA knockdown of the Egr2 inhibits the polarization of Macs towards both M1 and M2, and on the other hand, overexpression of Egr2 increased the polarization of M1 and M2 (202). Stimulation of Macs with IL-4 or IL-13 increases the Egr-2 expression (202). In order to test the role of indole on the expression of Egr-1 in BMDMs, Day 7 BMDMs from WT and AhR-KO mice were pretreated with indole or DMF solvent control overnight, then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS), and M2 (20ng/ml IL-4). Then (2x 10^5) cells stained extracellularly for CD11b and F4/80 as Mac markers and Egr-2 as M2 marker. Cells were analyzed using FACS. Percentage of Egr-2-positive cells is indicated on plots (Figure 25. A). mean fluorescence intensities (MFI) of the Egr-2-positive M2 population were measured (Figure 25. B). Our results indicated that indole reduces the expression of Egr-2 in M2 Macs, and the effects of indole are AhR independent. So far, our data has shown that indole reduces the expression of three M2 markers (CD206, Arg-1, and Egr-2) independent of the aryl hydrocarbon receptor. We have looked at the gene expression of few other M2 markers, such as (Chi313 and Relm-α), in the next sections.

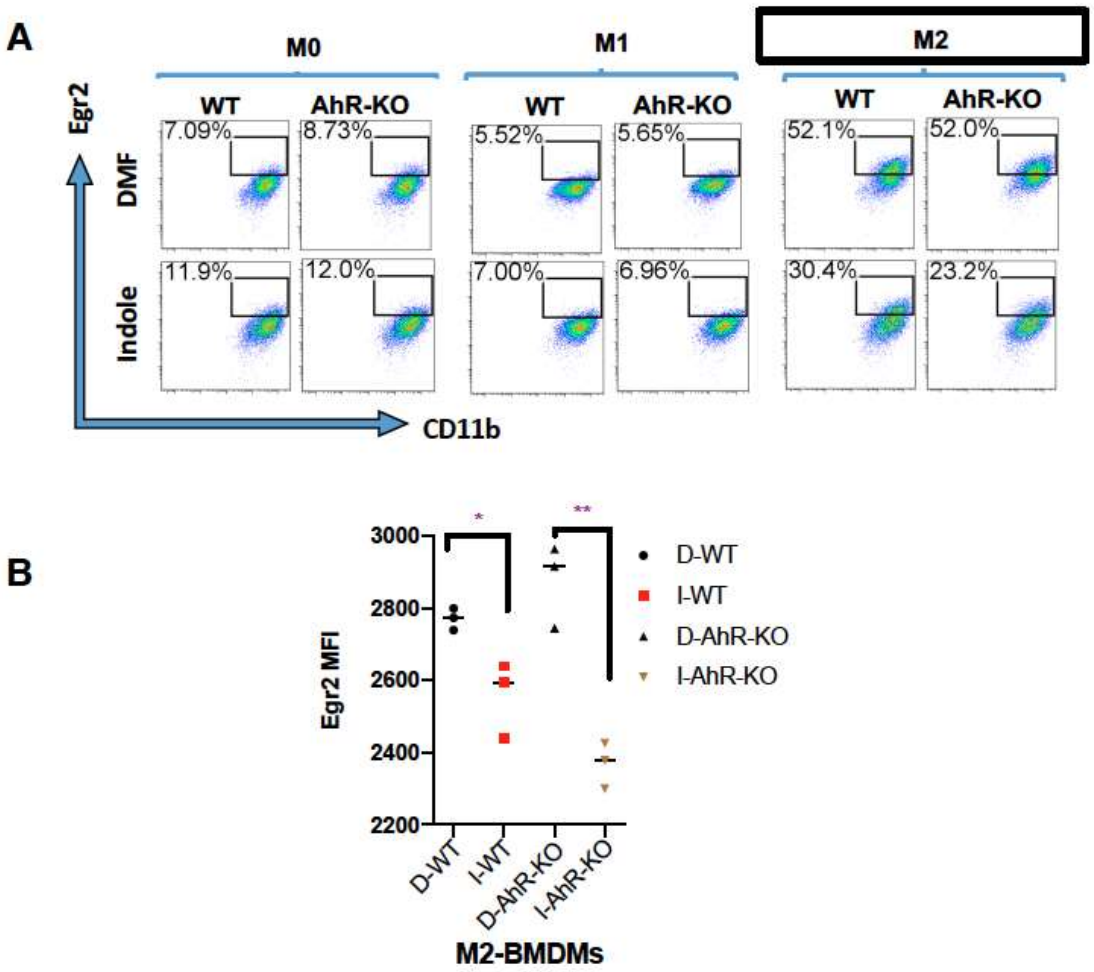


Figure 25. Indole decreases the expression of Egr-2 in M2 BMDMs, and the activity of indole is AhR independent.

Day 7 BMDMs were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS), and M2 (20ng/ml IL-4) (A) Flow cytometric analysis of BMDMs from WT or AhR-KO mice. (2x 105) cells stained extracellularly for CD11b and F4/80 as Mac markers and Egr-2 as M2 marker using FACS. Percentage of Egr-2-positive cells are indicated on plots. (B) mean fluorescence intensities (MFI) of the Egr-2-positive population. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Indole reduces the expression of Chi313 and Relm-α in M2 BMDMs

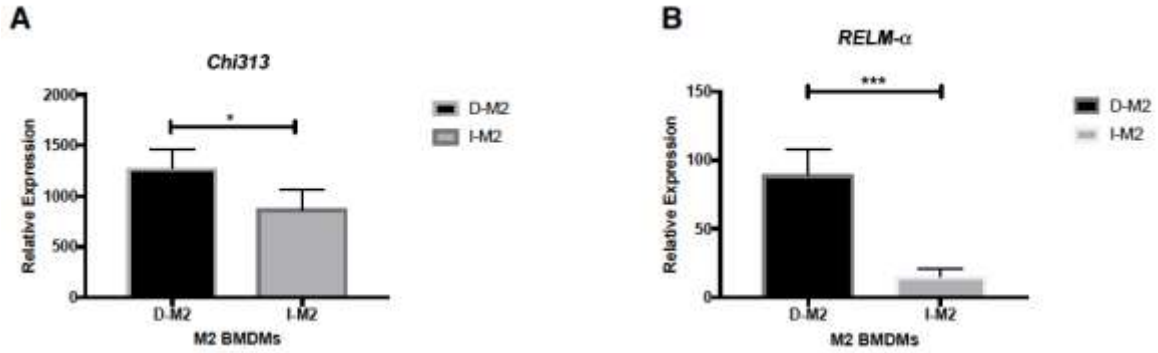
Chi313

Chitinase like lectin (CHI313), also named YKL-40 and cartilage glycoprotein 39 is a typical M2 marker. Chitinase like lectins are proteins from glycoside family 18 (203). Chi313 is expressed in different cell types such as neutrophils and Macs (204) . Expression of Chi313 has roles in regulating inflammation, tissue remodeling and fibril formation. In order to check the role of indole on Expression on Chi313 in M2 polarized BMDMs, day 7 BMDMs were pretreated with indole or DMF solvent control overnight then stimulated with polarization M2 (20ng/ml IL-4) skew for 24 hours. Then, relative gene expression of M2 marker, Chi313, measured using qRT-PCR (Figure 26. A). In line with previous M2 markers, Indole decreased the expression of Chi313. We continued to check the other M2 Mac markers such as Relm-α and PPAR-γ to better understand the role of indole in regulating M2 Macs.

Relm-α

RELM-alpha (Resistin-Like Molecule-alpha) is a part of a cysteine-rich C-terminus protein family called RELM (205). These proteins are conserved in mammals. RELM-α positive M2 Macs have been involved in both increase and decrease of the inflammation (206). To understand the role of indole in RELM-α expression of M2 polarized BMDMs, day 7 BMDMs were pretreated with indole or DMF solvent control overnight, then stimulated with polarization M2 (20ng/ml IL-4) skew for 24 hours. Then, the relative gene expression of the M2 marker, RELM-α, measured using qRT-PCR (Figure 26. B). our data show that indole decreased the expression of RELM-α similar to previously mentioned other M2 markers. Our data is suggesting that indole is not completely switching M1 polarized Macs to M2. Indole is probably inducing a new phenotype in Macs. The

regulatory roles of indole in Macs so far are AhR independent. This suggests that indole is regulating Mac metabolism by other pathways that are not dependent on AhR.



Day 7 BMDMs were pretreated with indole or DMF solvent control overnight then stimulated with

Figure 26. Indole reduces the expression of Chi313 and Relm-α in M2 BMDMs.

polarization M2 (20ng/ml IL-4) skew for 24 hours. Relative gene expression of M2 markers, Chi313 (A), and Relm-α (B) measured using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Indole increases the gene expression of M2 marker, PPAR-γ in polarized BMDMs independent of AhR

PPAR-γ

PPARs (Peroxisome proliferator-activated receptors) are transcription factors that have a role in the initiation and suppression of different genes' expression (207). PPAR-γ is induced by IL-4 or IL-13 and has a role in M2 polarization of Macs (208). Phosphorylated Stat-3, Stat-6, and AMPK are in cross-talk with PPAR-γ, (Figure 27. A right) in M2 Macs in contrast to M1 Macs that activate the PIK-AKT-mTORC-1 pathway (Figure 27. A left) (109). Our previous data (data not shown) suggested that indole increases the expression of the Stat-3 Macs. We also have data suggesting

that indole activated AMPK **(**data not shown**),** which may link AMPK to indoles regulation of metabolism. Data has shown that deficiency in PPAR-γ leads to reduced M2 polarization and increased levels of insulin signaling, obesity, and CVD (209). In order to test the role of indole on PPAR-γ in polarized BMDMs, day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight, then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of PPAR-γ, measured in polarized BMDMs using qRT-PCR. Our results show that in contrast with previously mentioned M2 markers, indole increases the PPAR-γ expression in BMDMs, especially M2 BMDMs (Figure 27. B). This data, along with our data regarding STAT-3 and AMPK (indole activates both) suggests that indole is not shifting the M1 polarization of Macs to M2, and instead, indole is regulating the polarization of Macs to a unique phenotype that is less inflammatory, yet with bacterial killing characteristics. Surface and intracellular markers specific to a certain phenotype of Mac are not the only way to distinguish Macs' polarization status. Activated metabolic pathways and functional properties of the polarized Macs can also give us strong clues about the specific phenotype of a Mac. In the next section, we will our functional results with regards to polarized Macs.

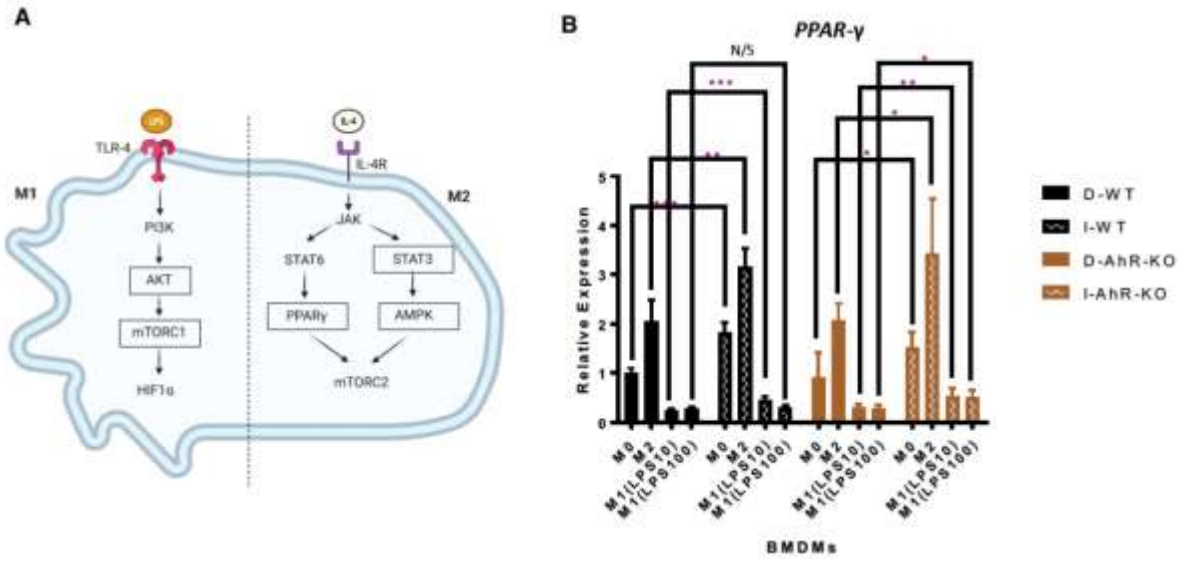


Figure 27. Indole increases PPAR-γ gene expression in BMDMs.

(A) Typical metabolism-related signaling pathways in polarized Macs. (B) Day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of PPAR-γ, measured in polarized BMDMs using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Energy production in the cell and glycolytic parameters

Glycolysis and mitochondrial Oxidative phosphorylation are the two major ATP production pathways in the cells (Figure 28. A) (97). Some cells have the capability to switch between these two pathways to meet their energy demands (108). The conversion of glucose to pyruvate in the cytoplasm is called glycolysis (97, 108). Pyruvate has two fates; it will either get converted to lactate or further metabolized through the TCA cycle and eventually electron transport chain (ETC) to produce more ATP (113). In order to get the maximal information about the glycolytic parameters of a cell, we minimize the OXPHOS pathways by using Oligomycin,

which is an inhibitor for mitochondrial ATP synthase or complex IV inhibitor. We also inject Rot/AA to completely shut down the ETC and get information about the maximal glycolytic capacity and the compensatory glycolysis in the cell (Figure 28. A) (210). Glucose is the primary fuel of the glycolysis (97). Since the XF assay medium doesn't have any glucose or glutamine, the first three measurements in the assay will be an indicator of the non-glycolytic acidification rate (Figure 28. B, C), which is indicative of acidification but not related to the conversion of glucose to lactate. After injection of glucose from port A, increased ECAR levels are indicators of Glycolysis rates (measurements 4,5 & 6). Next, by injection of Oligomycin from port B and injection of Rot/AA from port C, the ETC will be inhibited, and increased amounts of ECAR will be an indicator of Glycolytic capacity and the compensatory glycolysis rates of the cell (Figure 28. B&C). Compensatory glycolysis rates of BMDMs demonstrates the cellular energy management capability under mitochondrial stress conditions. In other words, this parameter indicates compensation for energy demand when mitochondrial respiration is inhibited. The last injection is 2 deoxy-glucose or 2DG from port D. 2DG is a competitive inhibitor of the glucose and negative control for the assay. 2DG will completely shut down the glycolysis pathway (211).

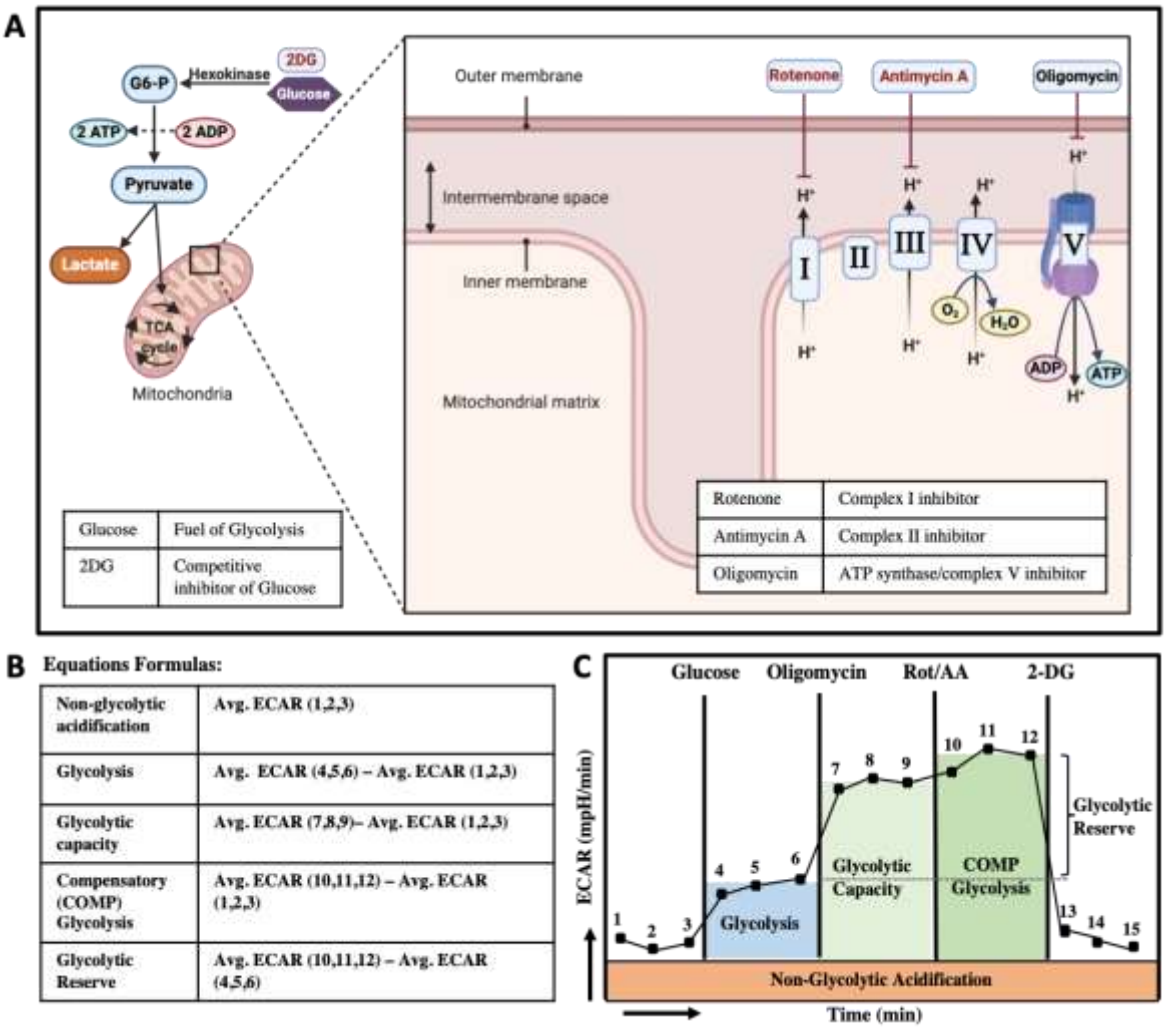


Figure 28. energy production in the cell and glycolytic parameters.

(A) schematic view of the two most important energy production pathways in the cell; Glycolysis (left) and mitochondrial oxidative phosphorylation (right). Glycolysis is the conversion of glucose to pyruvate. XF analyzer can detect the protons that are produced by the conversion of pyruvate to lactate as ECAR (mpH/min) levels. Inhibition of the ATP synthase followed by inhibition of complex I and II the in mitochondrial electron transport chain will eliminate the ATP production and proton efflux through OCR. (B) calculation of glycolytic parameters, (C) Glycolytic function parameters after each compound injection.

Glycolytic functions of naïve M0 and polarized M1 (LPS induced) and M2 (IL4 induced) BMDMs

Generally, polarized Macs have more glycolytic activities compared to naïve M0 Macs. LPS induced M1 Macs to possess the highest glycolytic activity. However, polarized Macs have more distinct separations in their OCR spare respiratory capacity (212), which has not shown in here, their glycolytic metabolism is also wholly distinguishable. It is important to note that an increase in ECAR in LPS induced M1 polarized BMDMs is not a definite characteristic for other types of M1 polarized Macs (such as LPS + IFN-g or PAMP induced M1s), and they may not increase or change the ECAR without glycolytic stress. As expected, In the first three measurements, which is the indicator of the non-glycolytic activity, polarized Macs do not show a significant difference because the media does not have any sources of glucose or pyruvate (Figure 29). After injection of glucose, polarized BMDMs indicate higher levels of glycolysis than naïve BMDMs, and M1 BMDMs demonstrate the highest levels of glycolysis compared to M0 and M1.

Typically, glycolytic metabolism is the preferred ATP production pathway in LPS induced M1 Macs, and OXPHOS is the main M2 ATP production pathway (97,212). After the injection of Oligomycin, the ATP synthase complex in the mitochondrial electron transport chain of BMDMs will shut down; thus, the cells will start to rely on glycolysis to meet their energy demands. Since glucose is present in the media, the glycolytic capacity of the polarized BMDMs will be comparable. Again, LPS-induced M1 BMDMs will have the highest glycolytic capacity. M2 and M0 will have lower glycolytic capacities, respectively (Figure 29. A, B). Injection of Rot/AA will

inhibit the Complex I and III of the mitochondrial ETC and completely shut down the OXPHOS, and there will be a slightly higher increase in the ECAR levels, which is an indicator of compensatory glycolysis. Again, M1 BMDMs will have the highest ECAR levels in this step; finally, 2 Deoxy Glucose (2DG), a competitive inhibitor of glucose and negative control for glycolysis, will completely shut down Glycolysis pathway.

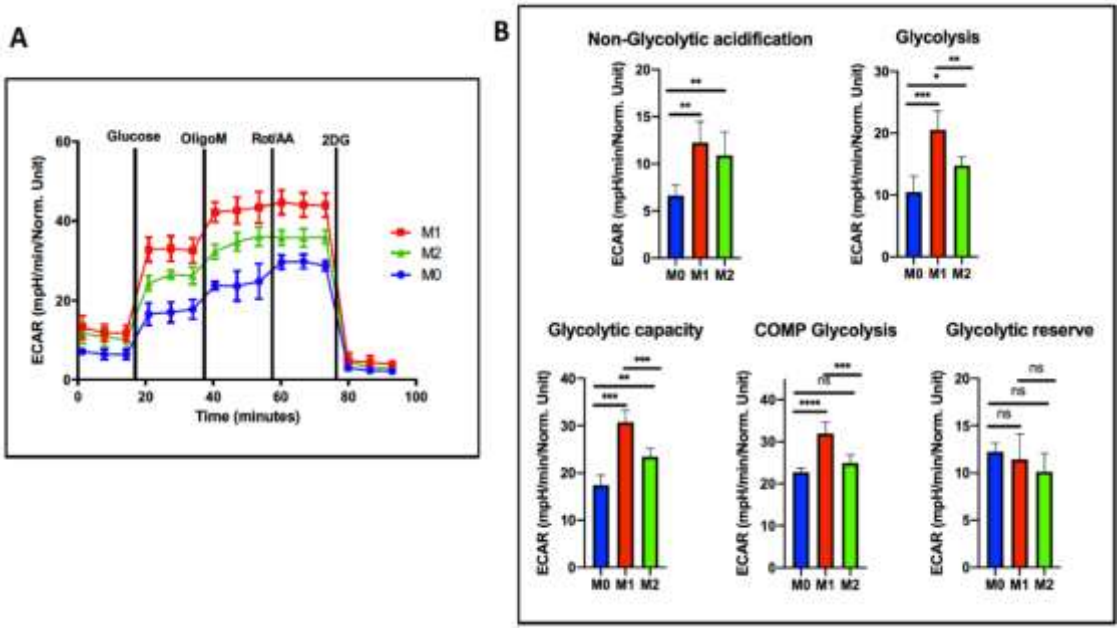


Figure 29. Glycolytic functions of naïve M0 and polarized M1 (LPS induced) and M2 (IL4 induced) BMDMs.

Glycolytic parameters of polarized Macs indicated as ECAR (mpH/min). (A) Non-glycolytic acidification rate, Glycolysis, Glycolytic capacity, and compensatory glycolysis as ECAR (mpH/min) in M0, M1, and M2 BMDMs. Injections of the ports are as follow: port A: Glucose, Port B: Oligomycin, Port C: Rotenone plus antimycin A and port D: 2 Deoxy Glucose (B) Bar graphs of each parameter for M0, M1, and M2 BMDMs. Data shown are from 4-6 culture wells per experiment. Measurements are based on means + SEM. Statistical significance between groups are based one-way ANOVA with Tukey's multiple comparison test at "*" $p < 0.05$, significance at "**" $p < 0.01$, significance at "***" $p < 0.001$, significance at "****" $p < 0.0001$.

Indole reduces glycolytic parameters in M1 BMDMs

To check the role of indole, in glycolytic parameters of M1 BMDMs, day 7 BMDMs were pretreated with indole or DMF solvent control overnight. Then the cells were exposed to 10ng/ml LPS for 24 hours as M1 skews. Then using the XF glycolysis stress kit, the glycolytic parameters of M1 BMDMs measured and indicated as ECAR (mpH/min), and indole was able to reduce (Figure 30. A) Glycolysis (Figure 30. B) Glycolytic capacity, and (Figure 30. C) Glycolytic reserve as ECAR (mpH/min) in M1 BMDMs. Injections of the ports were as follow, port A: Glucose, Port B: Oligomycin, and Port C: 2 Deoxy Glucose. Since Glycolysis is one of the important functional characteristics of M1 BMDMs, our results indicated that indole could be a good candidate for reducing extracellular acidification of the BMDMs exposed to M1 polarization skews.

Glycolytic parameters of polarized Macs indicated as ECAR (mpH/min). (A) Glycolysis, (B)

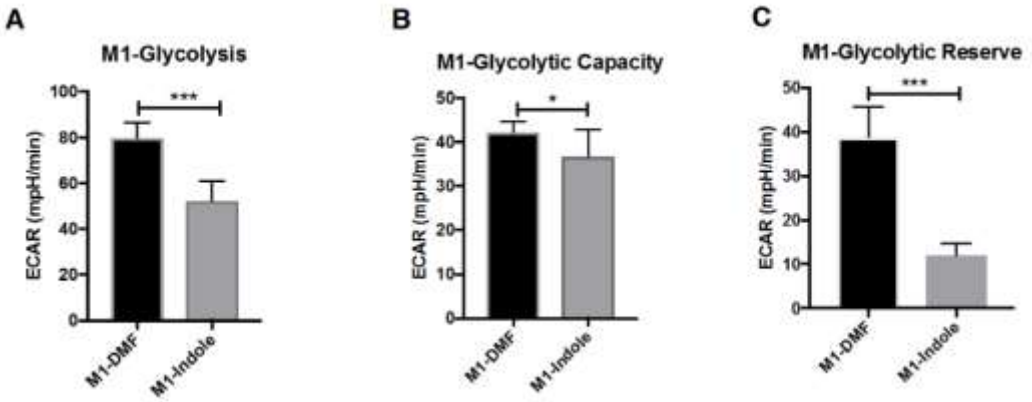


Figure 30. Indole reduces glycolytic parameters in M1 BMDMs.

Glycolytic capacity, and (C) Glycolytic reserve as ECAR (mpH/min) in M1 BMDMs. Injections of the ports are as follow, port A: Glucose, Port B: Oligomycin, and Port C: 2 Deoxy Glucose. Data shown are from 4-6 culture wells per experiment. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Indole reduced the lactate in cell culture media

Lactate is the final product of anaerobic glycolysis (213). Lactate is highly produced in activated Macs, especially in M1 polarized Macs (113). Since our glycolysis data from XF seahorse analyzer indicated that indole reduces glycolytic activities of BMDMs, as a complementary study we measured the lactate production of the day 7 BMDMs that are pretreated with indole and then polarized with M0 (Media), M1 (10ng/ml LPS) and M2 (20ng/ml IL-4) skews.

We quantified serum levels of Lactate with Lactate colorimetric/fluorometric assay kit (Sigma Aldrich); And analyzed the lactate quantification results based on (Figure 31. A) ng/µl and (Figure 31. B) nmol/µl scale. As expected, indole reduced the lactate production in polarized BMDMs, and this is in line with our seahorse Glycolysis-stress test data. Our data so far suggest that indole reduces the inflammatory or M1 like polarization of Macs, but it does not shift the polarization of Macs to a typical M2 polarized anti-inflammatory phenotype. But since in atherosclerosis, the main population of Macs in plaques is M1 Macs, reductions of M1 polarization by an endogenous metabolite can be a helpful candidate in the reduction of size and progression of atherosclerotic plaques.

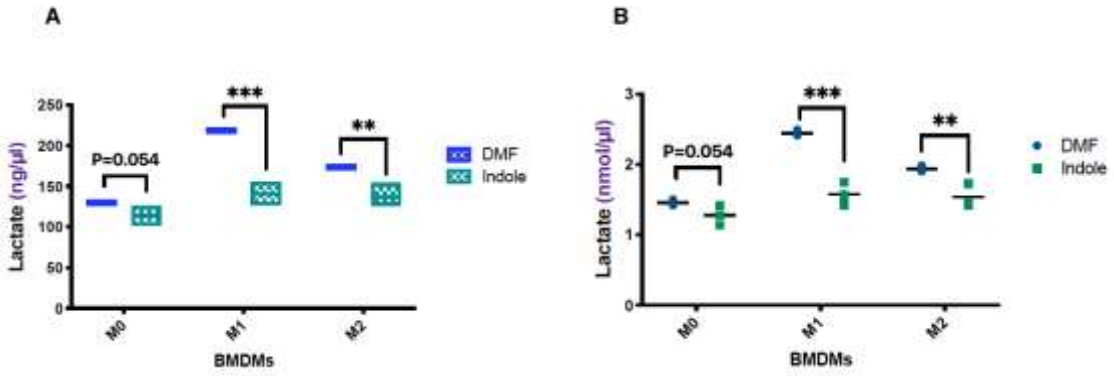


Figure 31. Indole reduced the lactate in cell culture media of BMDMs.

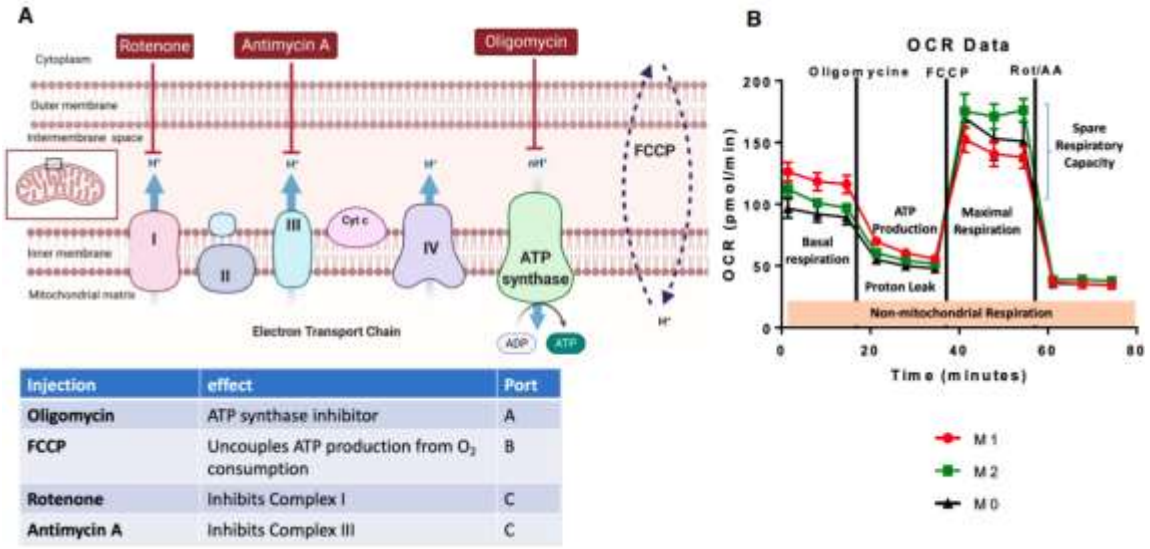
Day 7 BMDMs from WT mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) and M2 (20ng/ml IL-4). Lactate quantification with lactate colorimetric/fluorometric assay kit in (A)ng/µl and (B) nmol/µl scale. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Seahorse Mito-Stress test in BMDMs and validation of the techniques with the published data

Aerobic respiration is one of the leading ATP production pathways in the Macs that is more efficient than glycolysis (79). Active aerobic respiration is essential in activated M2 Macs. OXPHOS or Oxidative phosphorylation is composed of a series of electrons that enter into the mitochondrial electron transport chain (ETC) and, in sequence, pass through the complex I to V of the ETC. ETC develops a proton gradient in the intermembrane space of the mitochondria (Figure 2.21. A) and this proton gradient activates ATP synthase or complex V to produce ATP. Seahorse XF Mito-stress test measures the oxygen consumption rate (OCR) of the cells that is a representative of the mitochondrial function (respiration). Mito-Stress test of XF extracellular analyzers measures parameters related to mitochondrial function by injection of the modulators of the respiration and measuring OCR levels in real-time (212). There are 3 injections from 3 ports in this assay. Before injections, the first three measurements are an indicator of basal respiration.

Then after injection of Oligomycin, which is an inhibitor of ATP synthase, we can get information about ATP production of the cell. Port B (second injection) contains FCCP (Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone) which uncouples the oxygen consumption from ATP production, after the second injection we can get information about the maximal respiration of the cells and finally the last injection in port C contains Rotenone and Antimycin C that are respectively inhibitors of complex I and III of the mitochondria and completely shut down the mitochondrial activity, and we can gain information about the spare respiratory capacity of our cells (Figure 32. A, B). more information about the details of this experiment can be found in Agilent Seahorse XF Cell Mito Stress Test Kit User Guide #103015-100. Published data and my data indicate that when day 7 BMDMs are skewed with M1(LPS) and M2 (IL-4) skews, M2 Macs will have a higher spare respiratory capacity and M1 Macs will have the lower spare respiratory capacity (Figure 32. B). However, cell number, LPS, and IFN-gamma doses, duration of the experiment, Incubation time, solvent controls, normalization methods, and each injection dose can affect the results of the experiment. That is why, because of the sensitivity and variations of M1 polarization, we set up a titration study of M1 with different doses of LPS and IFN-γ (Figure 33). Since M1 polarization highly reduces the spare respiratory capacity ratios, we chose 10ng/ml LPS stimulation for our M1 polarization. Due to longer incubation of our BMDMs incubation, first with pretreatments (For example, with Indole of DMF solvent control), then with polarization skews, we chose a lower concentration to be able to detect the role of our pretreatment. The

polarization of the BMDM with lower doses of LPS was enough to induce M1 markers in FACS experiments.



Injection	effect	Port
Oligomycin	ATP synthase inhibitor	A
FCCP	Uncouples ATP production from O_2 consumption	B
Rotenone	Inhibits Complex I	C
Antimycin A	Inhibits Complex III	C

Figure 32. Seahorse Mito-Stress test in BMDMs and validation of the techniques with the published data.

(A) schematic view of mitochondrial oxidative phosphorylation. XF analyzer can detect the oxygen consumption rate (OCR). Rotenone is an inhibitor of Complex I of mitochondria. Antimycin A is an inhibitor of complex III of the electron transport chain (ETC). Oligomycin can inhibit ATP synthase or Complex V, and FCCP uncouples oxygen consumption from ATP production. (B) Day 7 BMDMs were pretreated with DMF solvent control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) and M2 (20ng/ml IL-4). (A) Oxidative phosphorylation parameters of polarized Macs indicated as OCR (pmol/min). Basal Respiration, ATP Production, Proton Leak, Maximal Respiration, and Spare Respiratory Capacity as OCR (mpH/min) in M0, M1, and M2 BMDMs. Injections of the ports are as follow, port A: Oligomycin, Port B: FCCP, Port C: Rotenone plus antimycin A and (B). Data shown are from at least four culture wells per experiment.

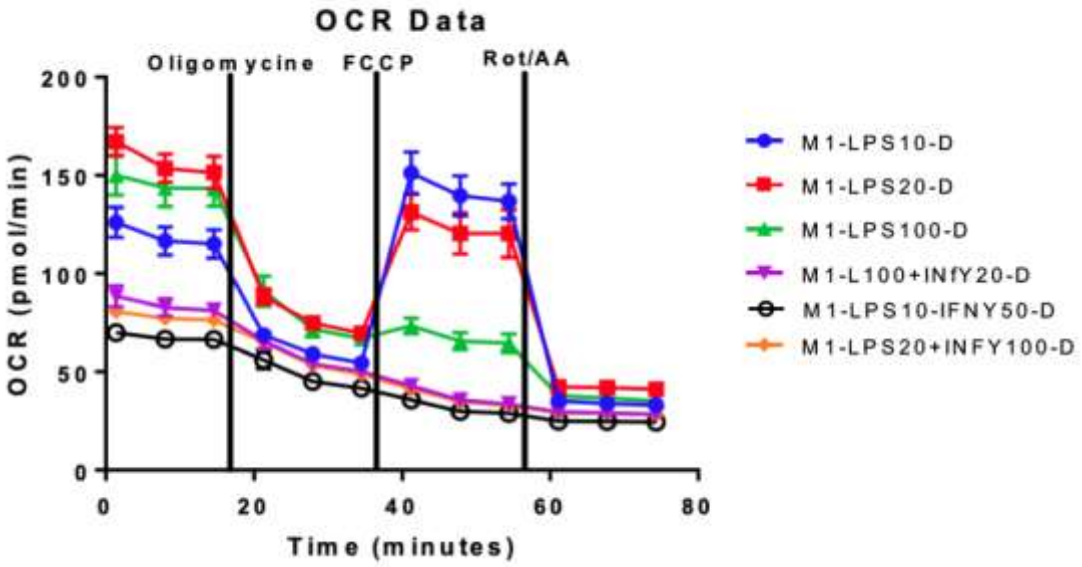


Figure 33. Oxygen Consumption Rate (OCR) of M1 BMDMs and titration of LPS and IFN-γ doses.

Day 7 BMDMs were pretreated with DMF solvent control overnight then stimulated with M1 polarization skews for 24 hours. Figure indicates the titration of M1 polarization with different doses of LPS and IFN-γ. Injections of the ports are as follow, port A: Oligomycin, Port B: FCCP, Port C: Rotenone plus antimycin A and (B). Data shown are from 4-6 culture wells per experiment.

BMDMs exhibit altered metabolic profiles after indole pretreatment

To check the role of indole on OCR and ECAR in polarized BMDMs, Day 7 BMDMs were pretreated with indole or DMF solvent control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) and M2 (20ng/ml IL-4). Oxidative phosphorylation parameters of polarized Macs were measured and indicated as OCR (pmol/min) (Figure 34. A). Injections of the ports are as follow, port A: Oligomycin, Port B: FCCP, Port C: Rotenone plus antimycin A. Then Glycolytic parameters of polarized Macs measured and indicated as ECAR (mpH/min) (Figure 34. B).

Injections of the ports are as follow, port A: Glucose, Port B: Oligomycin, Port C: 2 Deoxy Glucose. Data shown are from 4-6 culture wells per experiment. Our data results indicated that indole reduces the OCR and ECAR in all types of Macs, and this reduction in OCR and ECAR is independent of AhR, which means that indole using other pathways than AhR to induce its effects. Also, apparently, indole is activating other pathways in the cells to generate ATP. Our Hypothesis is that indole activates fatty acid oxidation (FAO) pathway and increases beta-oxidation as a source of ATP production in the cell. In the next sections (Chapter III), we have tested the role of indole in FAO and Beta- Oxidation.

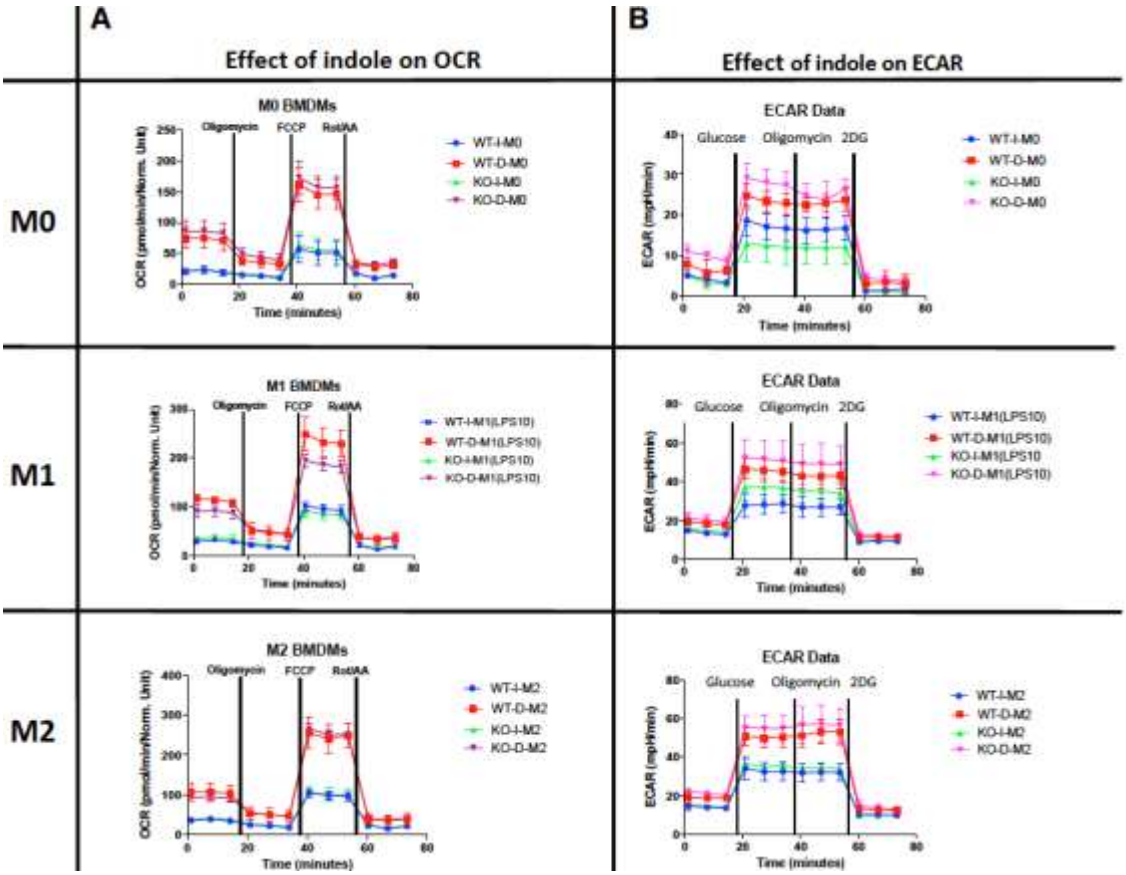


Figure 34. BMDMs exhibit altered metabolic profiles after indole pretreatment. Indole decreases the expression of the ATP5b subunit in M1-BMDMs.

Day 7 BMDMs were pretreated with indole or DMF solvent control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) and M2 (20ng/ml IL-4). (A) Oxidative phosphorylation parameters of polarized Macs indicated as OCR (pmol/min). Injections of the ports are as follow, port A: Oligomycin, Port B: FCCP, Port C: Rotenone plus antimycin A. (B) Glycolytic parameters of polarized Macs indicated as ECAR (mpH/min). Injections of the ports are as follow, port A: Glucose, Port B: Oligomycin, Port C: 2 Deoxy Glucose. Data shown are from 4-6 culture wells per experiment.

Indole decreases the expression of the ATP5b subunit in M1-BMDMs

ATP5b

ATP5b (ATP synthase beta subunit) gene encodes one of the subunits of the complex V of the mitochondria or ATP synthase (214). ATP synthase is the ATP producing subunit in the mitochondrial electron transport chain that leads to ATP synthesis using proton gradient during OXPHOS (215). ATP5b plays a role as a catalyst in ATP production (214). Indole induced reduction of OXPHOS in our polarized Macs made us interested in checking the role of indole on the expression of the ATP5b subunit. Since indole reduced OCR levels in mitochondria and reduced OXPHOS, we hypothesized that indole could decrease the function of ATP synthase, and lower levels of ATP5b is expected in indole pretreated BMDMS. In order to test this, Day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF solvent control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of ATP5b, measured in polarized BMDMs using qRT-PCR. Our results indicated that indole only reduced the expression of ATP5b only in M1 Macs (Figure 35). This can be because of the vulnerable activity of the OXPHOS in M1 Macs. The effects of indole on ATP5b are independent of AhR (Figure 35). In order to gain more information about the ATP production of Macs, we decided to perform a seahorse ATP assay.

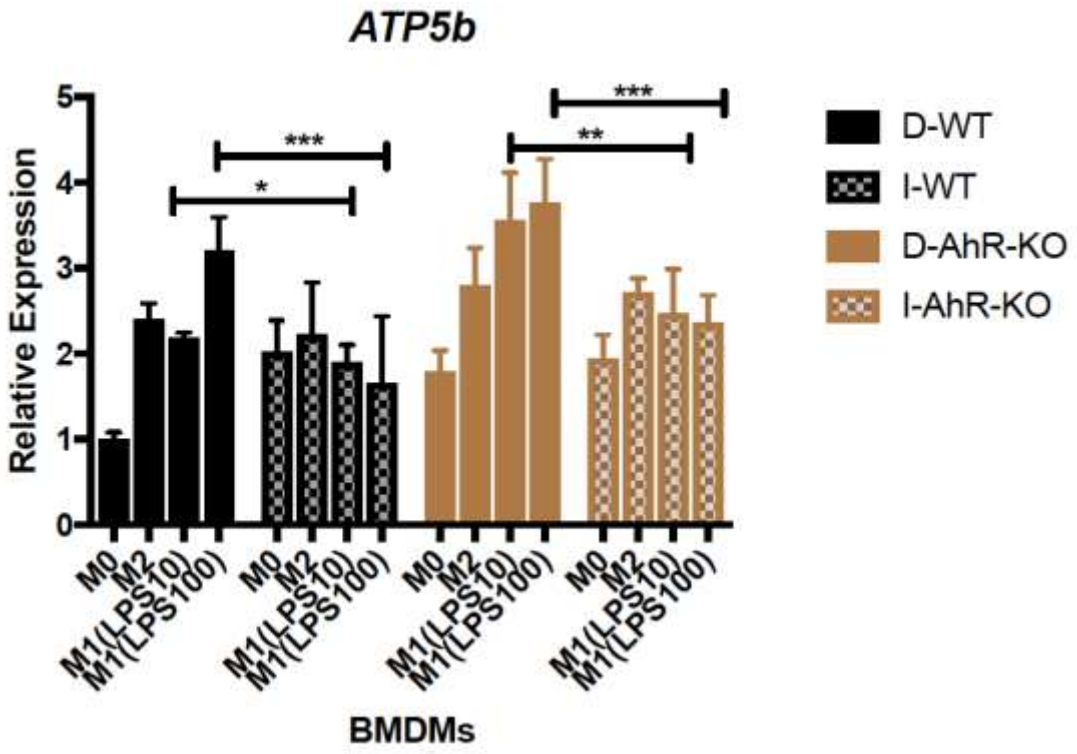


Figure 35. Indole decreases the expression of the ATP5b subunit in M1-BMDMs.

Day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of ATP5b, measured in polarized BMDMs using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Role of indole on ATP production of polarized Raw264.7 Macs

ATP or adenosine triphosphate is the most common energy-producing currency (intermediate metabolite) of the cells (216). ATP fuel comes from the breakdown of certain molecules in the cell (216). ATP is synthesized from inorganic phosphate and ADP (217). ATP production, ATP consumption, and ATP levels are different in various cells and their different states (156). Cells use a complex regulatory system to maintain ATP in a certain (steady) range,

and part of this regulation is through the ATP production (218). Decreased levels of ATP on the cells may reduce their viability (217-219). Information about ATP production and the ATP production source, which can be OXPHOS, glycolysis, or FAO, can be very informative about the energy demands and sources of the energy in the cell (97). Seahorse ATP assay measures the ATP production from glycolysis and mitochondrial oxidative phosphorylation. These are two of the major energy-producing pathways in the cells. However, it is important to mention that when fatty acids are the source of energy for the cell, the experiment results may be different. Fatty acid oxidation experiment has been explained in the next chapter. Most of the cells can switch their ATP producing pathways depending on their microenvironment and their activity states (97, 212). Since OXPHOS consumes oxygen, OCR's oxygen consumption rate will be an indicator of the OXPHOS pathway. On the other hand, the conversion of glucose to lactate through glycolysis leads to proton production per lactate. This causes the extracellular acidification, and ECAR levels will indicate glycolysis pathways in the cell. Part of the extracellular acidification can be through TCA Cycle as well. This assay will show us the amount of ATP production on our polarized Macs, and it will give us information about the ratio of ATP production through each pathway (219). The ATP assay report generator will produce the basal ATP production and stressed ATP production of the cells. Calculation formulas are provided in the methods section. To check the role of indole in the polarized Macs, Raw264.7 cell lines were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) and M2 (20ng/ml IL-4). ATP Rate Index was measured based on the mitochondrial ATP production to glycolysis ATP production ratios (Figure 36. A). In bar graphs of basal ATP Production Rate, the Blue section of the bar graph is an indicator of mitochondrial energy production, and the red section is an indicator of glycolytic ATP production (Figure 36. B). Energetic Map of ATP

production was measured in polarized Macs that were pretreated with indole or DMF solvent control (Figure 36. C)**.**

Our data indicated that, generally, indole is reducing ATP production through glycolysis and OXPHOS. And M1 Macs demonstrate the lowest Mito-ATP to glyco-ATP ratios, and M2 Macs indicate the highest MIT-ATP to glycol-ATP ratios (Figure 36. A). in basal ATP production ratios, M1 Macs indicate the highest Glyco-ATP production rate, and generally, M1 Macs have the highest ATP production ratios. And in all types of Macs indole is reducing both Mito-ATP and Glyco-ATP production (Figure 36. B). Energy map of the basal energy production rate of solvent control pretreated polarized Macs vs. indole pretreated group indicated that indole pretreated Macs have significantly lower energy production rates with glycolysis and OXPHOS ((Figure 36. C), when glucose is present in the cell media. Our results suggest that indole is probably activating another ATP producing pathway in the cells. The fatty acid oxidation pathway is another pathway that will be measured and discussed in the next chapter.

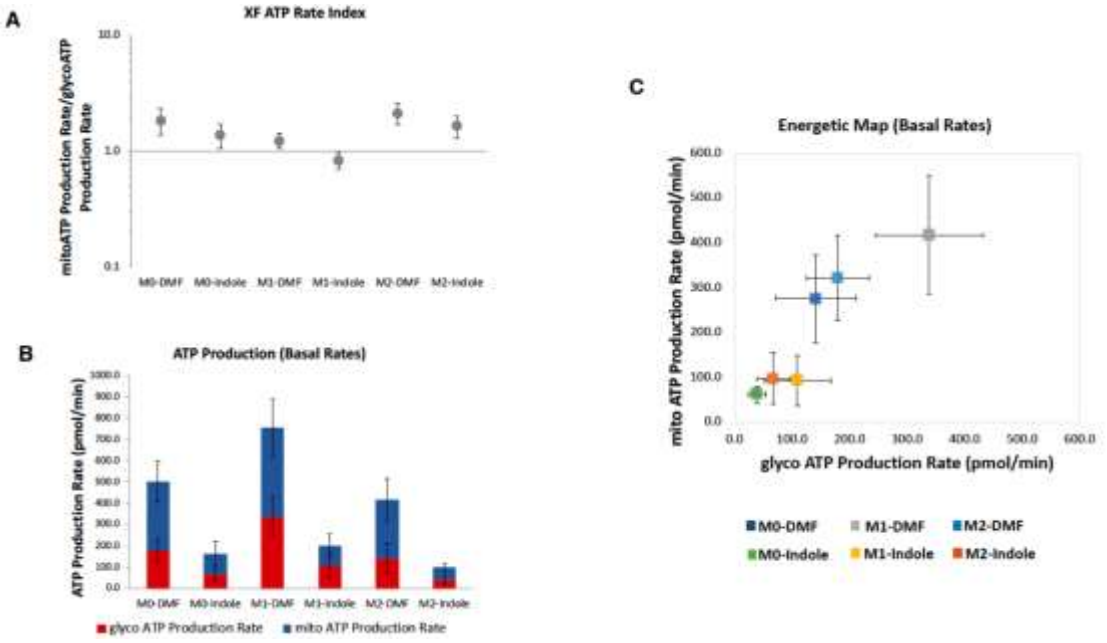


Figure 36. Role of indole on ATP production of polarized Raw264.7 Macs.

Raw264.7 cell lines were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) and M2 (20ng/ml IL-4). The Agilent Seahorse XF ATP Real-Time rate assay was performed to measures and quantify the rate of ATP (adenosine triphosphate) production from glycolysis and mitochondria simultaneously. (A) Seahorse XF Real-Time Assay, ATP Rate Index (B) Seahorse XF Real-Time Assay, Basal ATP Production Rate. (C) Seahorse XF Real-Time Assay, Energetic Map.

Indole does not prevent the production of mitochondrial ROS and the accumulation of dysfunctional mitochondria in BMDMs

Our previous lab data indicated that indole does not change NO and ROS production in DCs but Since we had increased levels of indole induced iNOS in Macs, and also, we observed that indole reduces ATP production, we set up to examine the mitochondrial ROS levels. We used day 7 WT BMDMs pretreated them with DMF (Solvent control) or 1mM Indole, or 1μM Oligomycin (positive control) for 24 hours and then stained the cells with MitoSOX which is an

indicator of mitochondrial superoxide levels along with Mito-Tracker green, which is an indicator of mitochondrial mass (220). Our data demonstrated that indole pretreated BMDMs indicated more ROS-producing mitochondria. This results maybe associated and in line with bacterial killing inducing properties of indole (Figure 37. A).

In order to check whether the increased mitochondrial ROS levels and reduction of ATP production is due to accumulation of dysfunctional mitochondria with reduced mitochondrial membrane potential, we used mito-tracker green which is a stain that is independent of mitochondrial membrane potential and along with mito-tracker red which is a stain dependent on mitochondrial membrane potential. Mito-tracker green is an indicator of mitochondrial mass and mito-tracker red is an indicator of mitochondrial function. MitoTracker $Green_{+high}$ with $MitoTrackerRed_{+low}$ expression is an indicator of dysfunctional mitochondria (220). Our results indicate that indole increases dysfunctional mitochondria compared to DMF solvent control (Figure 37. B). In this study, Oligomycin used as a positive control. Indole induced increased amounts of dysfunctional mitochondria may be associated with increased levels of ROS production. ROS can be involved in bacterial killing process of Macs (221). Our results so far have indicated that indole probably induces bacterial killing properties (data not shown) through increased iNOS production. Also, ROS production can be another factor associated with bacterial killing properties of indole.

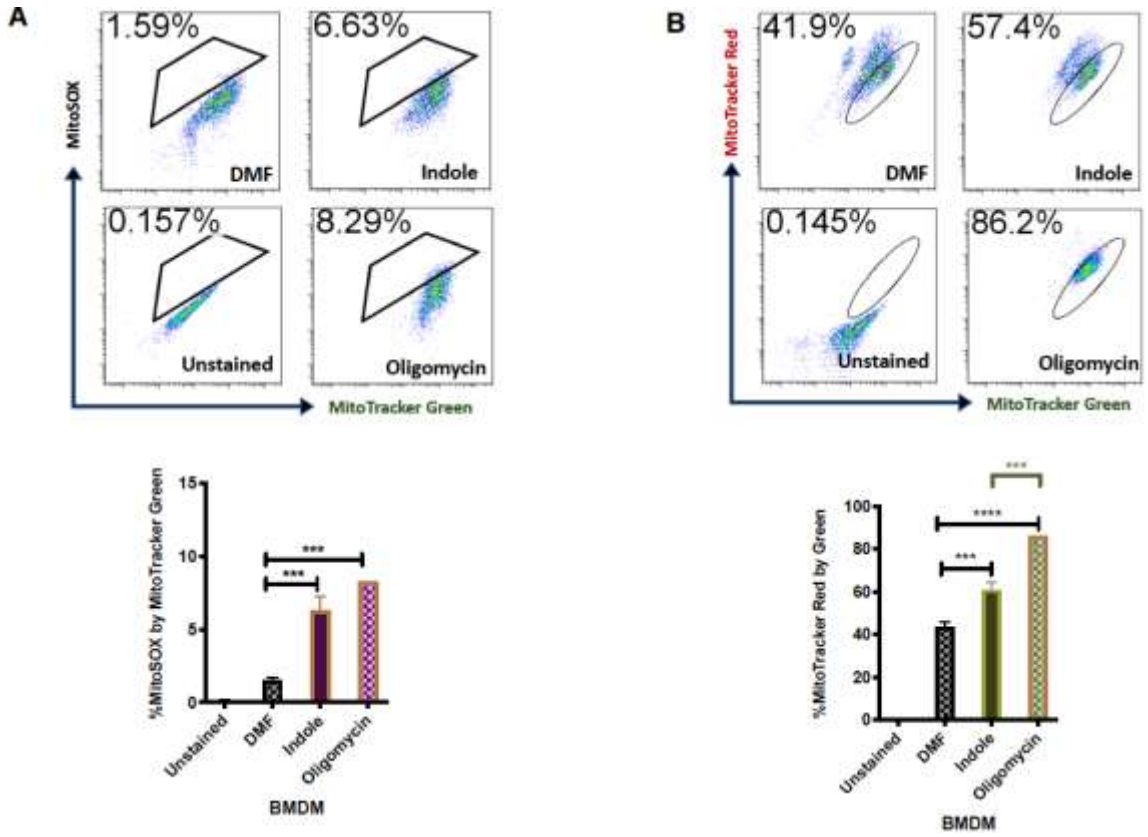


Figure 37. Indole does not prevent the production of mitochondrial ROS and the accumulation of dysfunctional mitochondria in BMDMs.

Day 7 WT BMDMs pretreated with DMF (Solvent control), 1mM Indole, or 1µM Oligomycin (control) for 24 hours. Total mitochondrial mass was analyzed by flow cytometry in cells labeled with MitoTracker Green (A). ROS and mitochondrial membrane potential were investigated in cells labeled with MitoTracker Green and MitoSOX (B), or with MitoTracker Red, respectively.

Contribution of NAD+/NADH in cellular metabolism.

Many physiological and cellular processes are coordinated by signaling pathways. For Example, pathways such as migration, senescence, apoptosis, etc. are coordinated by molecular signaling pathways (222). Glucose, lipids**,** and protein metabolisms are perceived as primary molecular pathways in controlling the cell metabolism and complex structures (223). however, simple chemical small molecules such as NAD (Nicotinamide adenine dinucleotide) and NADH

(Nicotinamide adenine dinucleotide + hydrogen (H) are also important molecules in the regulation of metabolism and linking the important metabolic pathways to each other (224).

The transformation of NAD from NAD+ (its oxidized form) to NADH (reduced form) and vice versa, provides the cell with a mechanism for accepting and donating electrons (225). NAD+/NADH has a significant role in the reactions regarding glycolysis, TCA Cycle, and β-Oxidation (Figure 38.) (225).

Indole Increases NADH gene expression in all polarized BMDMs independent of AhR and increases NAD in M1 BMDMs

In order to check the role of indole in regulating NADH expression, we used day 7 BMDMs from WT or AhR-KO mice, and then we pretreated them with indole or DMF solvent control overnight. Then, the cells stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of NADH measured in polarized BMDMs using qRT-PCR (Figure 39. A). Our data indicated that indole increased NADH gene expression in all types of polarized BMDMs independent of AhR. The data suggest that indole pretreatment increases NADH expression to increase the regulation of pathways that need NADH.

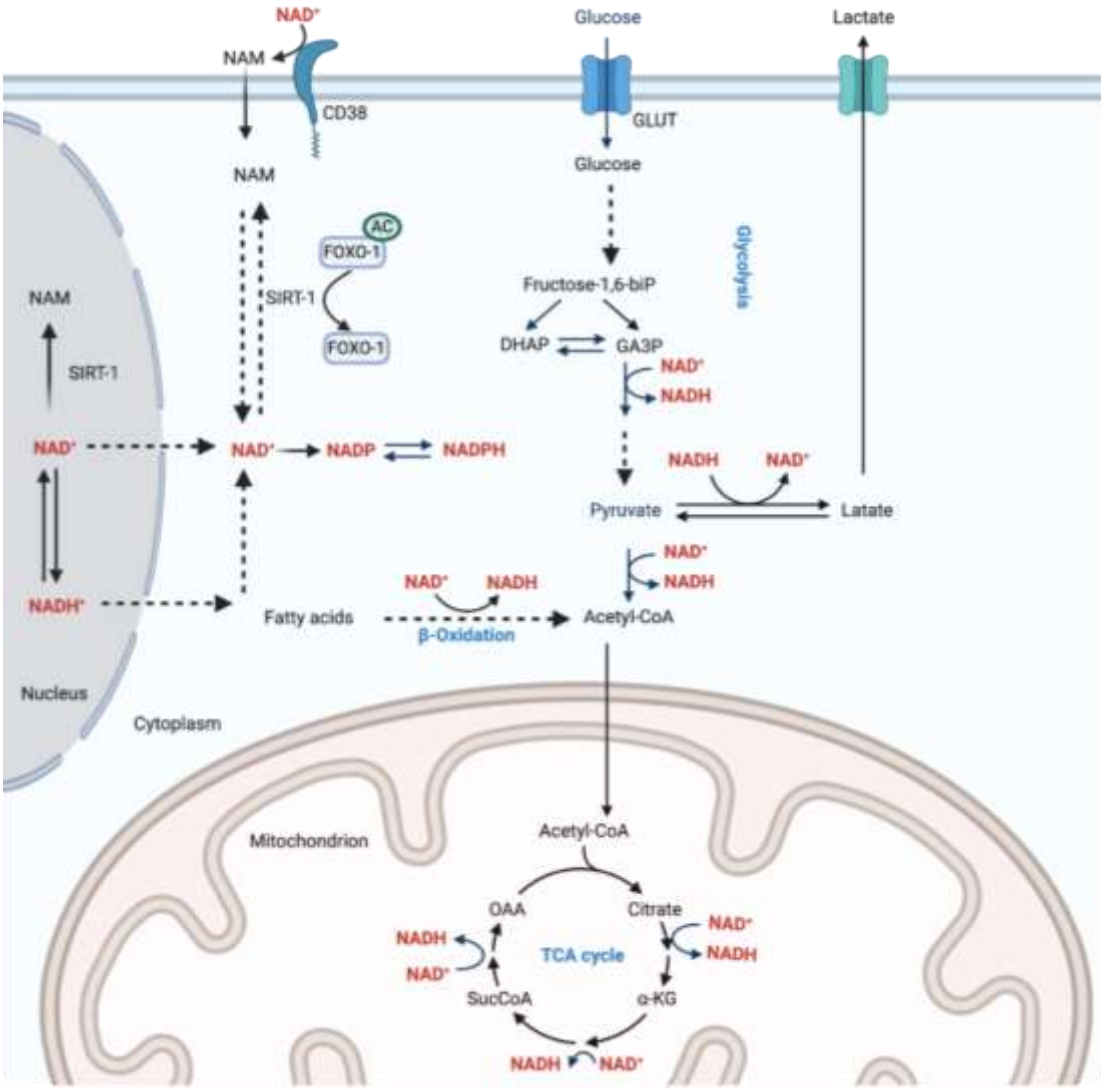


Figure 38. Contribution of NAD+/NADH in cellular metabolism.

The transformation of NAD from NAD+ (its oxidized form) to NADH (reduced form) and vice versa, provides the cell with a mechanism for accepting and donating electrons. NAD+/NADH has a significant role in the reactions regarding glycolysis and β-Oxidation. NAD, Nicotinamide adenine dinucleotide, NADH, Nicotinamide adenine dinucleotide + hydrogen (H); NADP, Nicotinamide adenine dinucleotide phosphate; NMN, nicotinamide mononucleotide, a competitor of NAD+ and alternative substrate of CD38; CD38, Cluster of differentiation 38, one of the main cellular NADases in mammalian tissues; SIRT1, Sitrulin 1, an enzyme that deacetylates proteins that contribute to cellular regulation; FOXO, Members of the class O of forkhead box transcription factors.

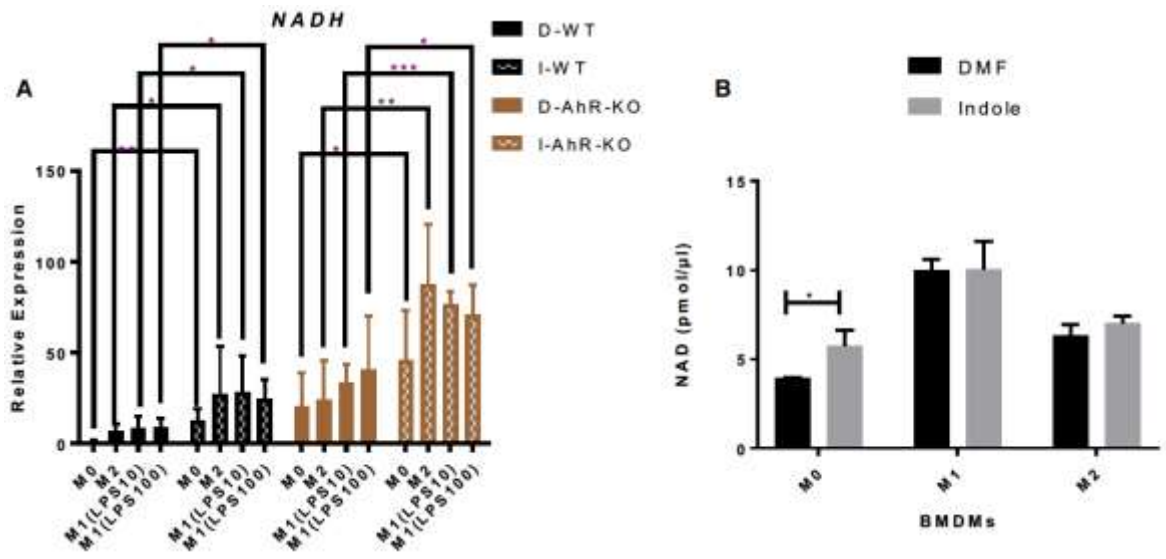


Figure 39. Indole Increases NADH gene expression in all polarized BMDMs independent of AhR and increases NAD in M1 BMDMs.

(A) Day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of NADH measured in polarized BMDMs using qRT-PCR. Data are representative of one of three experiments. (B) Polarized day 7 BMDMs NAD quantification with NAD colorimetric assay kit. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

NADH and NAD+ are both types of the coenzyme nicotinamide adenine dinucleotide (225). NADH is the reduced form, and NAD+ is the oxidized form. One of the essential parts of the redox reactions is the electrons transferring that converts NAD+ into NADH and vice versa (224,225).

We also measured the intracellular NAD+ levels in polarized Macs. We quantified NAD levels of polarized BMDMs with the NAD colorimetric assay kit (AMSBIO). Our data indicated that indole increased the NAD levels in M0 Macs (Figure 39. B).

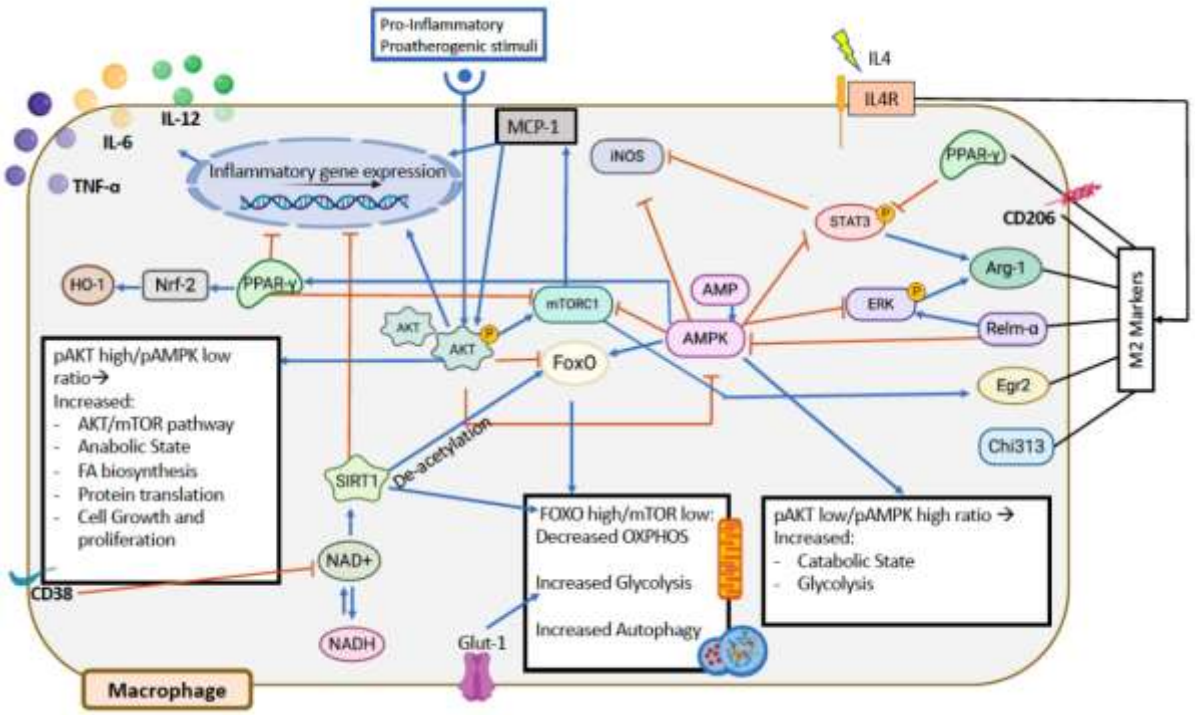


Figure 40. Important Immuno-metabolic pathways related to foam cell formation.

Immuno-metabolic pathways that are important in the process of Mac to foam cell formation. In atherosclerotic plaques, most of the Macs are stimulated with proatherogenic/pro-inflammatory signals (LPS, ox-LDL, etc) that polarize them towards the M1 phenotype. However, M2 Macs play a role in the stability of the plaques, and they get polarized with anti-inflammatory cytokines such as IL-4 or IL-10. ATGL, adipose triglyceride lipase; PPAR, peroxisome proliferator-activated receptor; NAD, Nicotinamide adenine dinucleotide; NADH, Nicotinamide adenine dinucleotide + hydrogen (H); NADP, Nicotinamide adenine dinucleotide phosphate; NMN, nicotinamide mononucleotide, a competitor of NAD+ and alternative substrate of CD38; CD38, Cluster of differentiation 38, one of the main cellular NADases in mammalian tissues; SIRT1, Sitrulin 1, an enzyme that deacetylates proteins that contribute to cellular regulation; FOXO, Members of the class O of forkhead box transcription factors; MCP-1, The monocyte chemoattractant protein-1; iNOS, Inducible nitric oxide synthase; STAT3, signal transducer and activator of transcription 3; Arg-1, Arginase 1; Relm-α, Resistin-like molecule alpha; Egr2, Early growth response protein 2; CD206, mannose receptor and C-type lectin; Chi313, chitinase-like lectin; ERK, extracellular signal-regulated kinase; AMP, adenosine monophosphate; AMPK, 5' AMP-activated protein

kinase; mTORC1, mammalian target of rapamycin complex 1; AKT, Protein kinase B (PKB); Nrf-2, nuclear factor erythroid-derived 2-like 2; HO-1, Heme oxygenase-1; Glut-1, Glucose transporter 1.

Studies have shown that gene or pharmaceutical restoration of NAD+ can reduce the metabolic decline in the cell, and NAD+/ SIRT pathway can regulate the cell metabolism by activating FOXO signaling (Figure 38) (226). There are many metabolic pathways in the cell in the process of Mac to foam cell formation that are all connected to each other. Upregulation and downregulation of each pathway can have a role in the progression or inhibition of foam cell formation (Figure 40).

Immuno-metabolic pathways that are important in the process of Mac to foam cell formation are depicted in Figure 40. In atherosclerotic plaques, most of the Macs are stimulated with proatherogenic/pro-inflammatory signals (LPS, ox-LDL, etc.) that polarize them towards the M1 phenotype. However, M2 Macs play a role in the plaques' stability, and they get polarized with anti-inflammatory cytokines such as IL-4 or IL-10 (132). Understanding the characteristics of the pathways and mechanisms related to formation in atherosclerosis is important to understand lipid homeostasis in foam cells better and discover novel therapeutic interventions to prevent atherosclerosis (Figure 40). We have tested the role of indole in regulating the pathways mentioned in Figure 40.

Indole increases SIRT-1 expression in BMDMs independent of AhR

SIRT-1

SIRT-1 or Sirtulin 1 is a conserved NAD-dependent protein deacetylase that contributes to cellular regulation. SIRT1 has an essential role in the pathogenesis or prevention of atherosclerosis (227). Data has shown that inhibition of SIRT1 can increase plaque formation, increase the Macs

infiltration (by increasing MCP-1) in the plaques and reduce Atg5-protein autophagy in Macs (228). To measure role of indole on SIRT1 in Macs, day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Then relative gene expression of SIRT-1 measured in polarized BMDMs using qRT-PCR. Our data indicated that indole increases SIRT-1 expression in polarized BMDMs independent of AhR (Figure 41). These data are in line with our NAD data and suggest that indole may have a role in regulating other pathways. FOXO and SIRT1 interact with each other in various pathways to regulate metabolism and their interaction has been associated with regulation of pathways involved in atherosclerosis and aging metabolism (229). In the next section we have looked at the role of indole on regulation of FOXO.

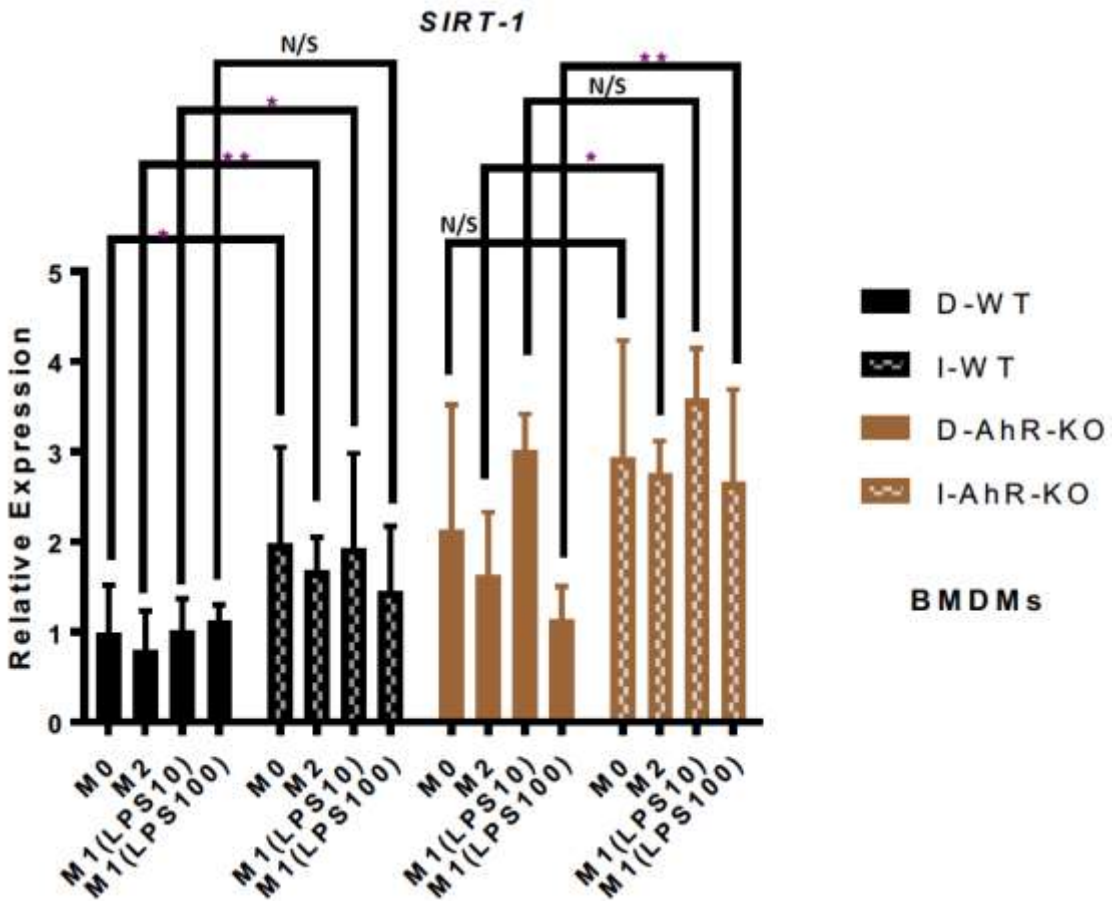


Figure 41. Indole increases SIRT-1 expression in BMDMs independent of AhR.

Day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of SIRT-1 measured in polarized BMDMs using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Indole increases FOXO-1 expression in BMDMs independent of AhR

FOXO1

FOXO1 or Forkhead box protein O1 is a transcription factor involved in regulating multiple energy metabolism pathways (230). FOXO has a role in regulating autophagy pathways and has

roles in regulating atherosclerosis pathways (231). FOXO1 may have a role in switching energetic carbohydrate pathways of the cell to the fatty acid oxidation pathway as a main energy producing path (232). To check the role of indole on regulation of FOXO-1 in Macs, day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight, then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of FoxO, measured in polarized BMDMs using qRT-PCR. Our results indicated that indole increases the FOXO1 expression only in the M0 and M1 (100ng/ml LPS) group (Figure 42). The effects of indole in regulation of FOXO in Macs were independent of AhR. Indole did not change the expression of FOXO1 in M2 and M1 (100ng/ml LPS) groups. Since indole was capable of increasing FOXO1 at least in M0 and M1 Macs, it may be able to regulate other pathways that are linked to Mac to foam cell formation related pathways.

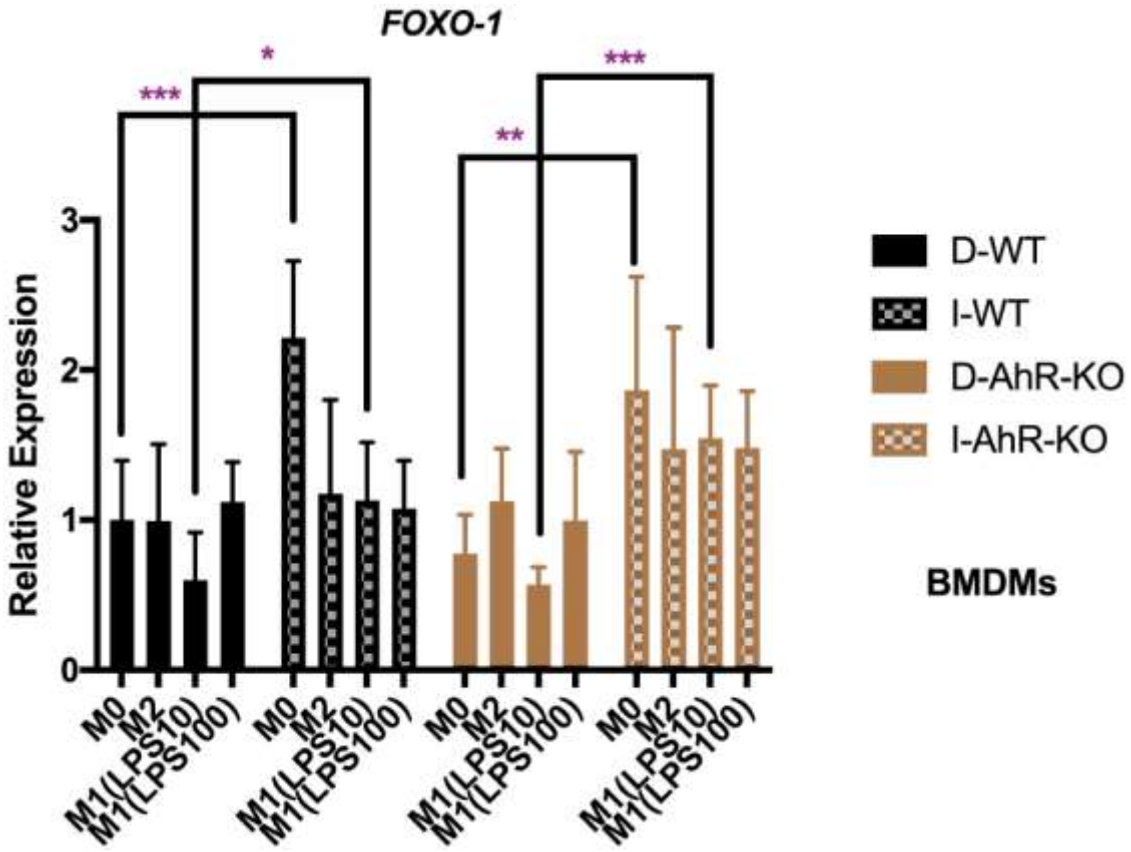


Figure 42. Indole increases FOXO-1 expression in BMDMs independent of AhR.

Day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of FoxO, measured in polarized BMDMs using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Nrf2/HO-1 pathway

Nrf2

Nrf2 or nuclear factor erythroid 2-related factor 2 is a basic protein (transcription factor) that has a role in the expression of antioxidant proteins (233).

Nrf2 is naturally found in the body, and it protects against oxidative damage derived by injury or inflammation (234). PPAR- γ upregulates Nrf-2 and has a critical role in the regulation of inflammation (235). PI3/AKT pathway activation can upregulate Nrf2 (Figure 43) (234, 235). Nrf-2 can protect the body against free radicals (234). Free radicals are involved in the transformation of LDLs to ox-LDLs (236), so Nrf-2 can indirectly reduce the foam cell formation.

HO-1

HO-1 is a Nrf2-regulated gene that has an important role in the prevention of vascular inflammation and the production of anti-inflammatory cytokines such as IL-10 (Figure 43) (237).

Indole slightly increases Nrf-2 in polarized BMDMs and does not change HO-1 in polarized BMDM

Because of the aforementioned reasons, we became interested in checking the role of indole on the regulation of Nrf2 and HO-1. In order to check this, day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of Nrf-2 (Figure 44. A) and HO-1 (Figure 44. B), measured in polarized BMDMs using qRT-PCR. Our results indicated that indole increases the expression of

Nrf-2 in polarized BMDMs independent of AhR (Figure 44. A). But on the other hand, indole does not change the expression of HO-1 in WT and AhR-KO BMDMs (Figure 44. B).

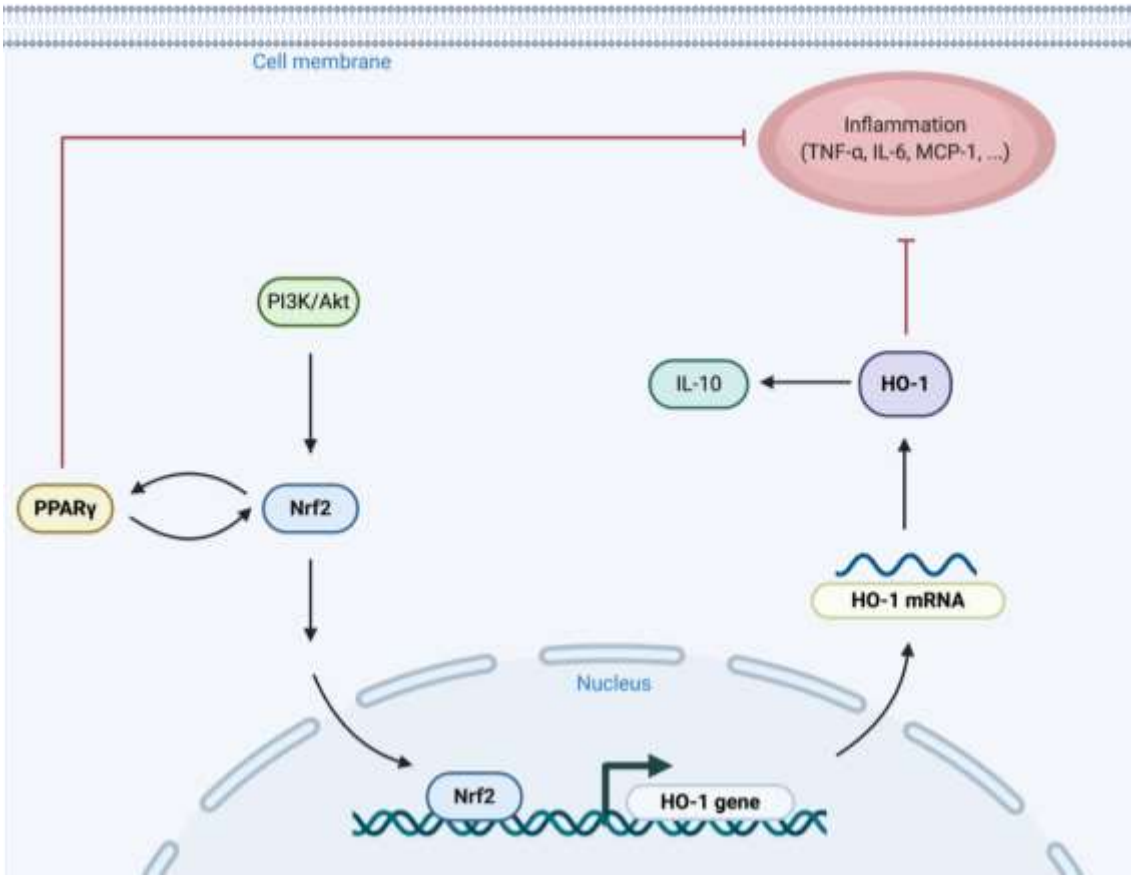


Figure 43. Simplified Nrf2/HO-1 pathway.

HO-1 is a Nrf2-regulated gene that has an important role in the prevention of vascular inflammation and production of anti-inflammatory cytokines such as IL-10. Nrf2 is a basic protein that has role in the expression of antioxidant proteins that protect against oxidative damage derived by injury and inflammation in the cell. PPAR-g upregulates Nrf-2 and has a critical role in regulation of inflammation. PI3/AKT pathway activation can upregulate Nrf2. HO-1, heme oxygenase-1; Nrf2, nuclear factor erythroid 2-related factor 2; PPAR, peroxisome proliferator-activated receptor; IL-10, Interleukin 10; PI3K, Phosphoinositide 3-kinases; Akt, protein kinase B (PKB); TNF, Tumor necrosis factor; IL-6, Interleukin 6; MCP-1, The monocyte chemoattractant protein-1.

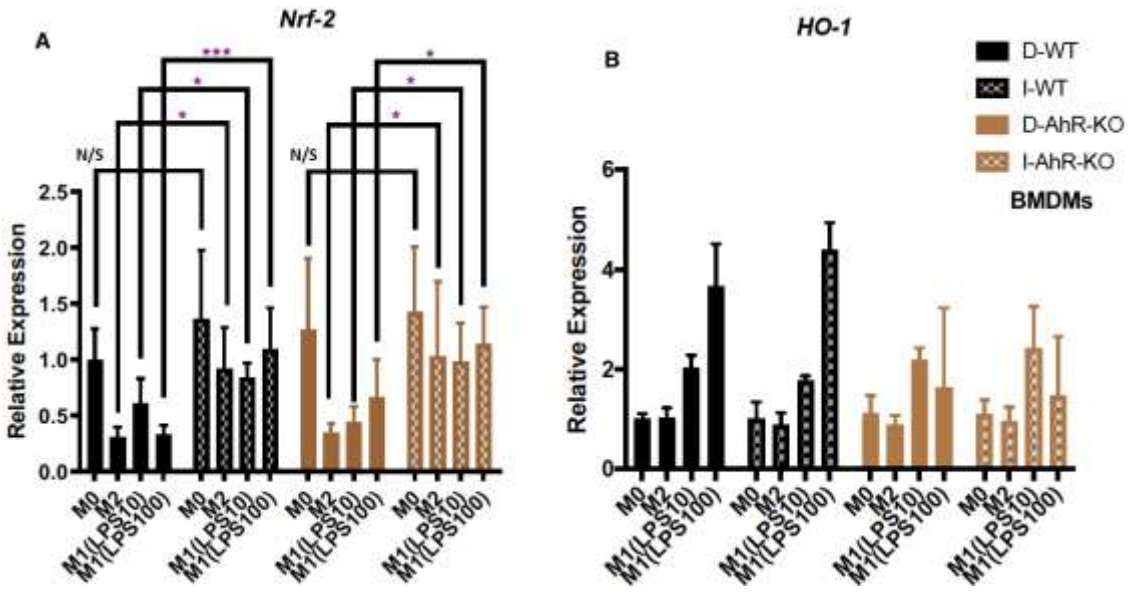


Figure 44. Indole slightly increases Nrf-2 in polarized BMDMs and does not change HO-1 in polarized BMDM.

Day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of (A) Nrf-2 and (B) HO-1, measured in polarized BMDMs using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Indole's effects on Mac gene, protein, function, and dependency on AhR.

So far, our data has indicated that indole can regulate many pathways that have a role in foam cell formation. Table 1 is a color-coded list of the genes, proteins, and cell functions in the Macs that we have tested (some listed information is from previous lab data) the role of indole on their regulation. We have also mentioned the indole's regulation dependency on AhR.

Table 1. Table of indole's effects on Mac gene, protein, function and dependency on AhR

Immuno-metabolism

Color coded role of indole:
Increase: M0, M1, M2
Decrease: M0, M1,M2
No Change: M0, M1,M2

Immuno-metabolism	Gene	Protein	Function	Dependency on AhR
IL-12		M0		Independent
TNF-a		splenic Macs		Independent
		Palmitate stimulated M0		
		splenic MQ		Independent
P-Stat3		M0		Independent
IL-6		M0		
pAKT		M0		
pERK		M0		
CD38	M1	M0, M1, M2		Independent
iNOS	M1	M0, M1, M2		Independent
MCP-1	M1			Independent
CD-206	M2	M0, M1, M2		Independent
Arg-1	M2	M0, M1, M2		Independent
Egr2		M0, M1, M2		Independent
Relm-α	M2			
Chi313	M2			
PPAR-γ	M0, M1, M2			Independent
Glycolysis			M0, M1, M2	Independent
OXPHOS (SRC)			M0, M1, M2	Independent
ATP5b	M1			Independent
NAD		M0,M1,M2		
NADH	M0, M1, M2			Independent
SIRT-1				Independent
FOXO-1	M0, M1,M2			Independent
Glut-1	M0, M1, M2			Independent
HO-1	M0, M1, M2			Independent
Nrf-2	M0, M1, M2			Independent

Indole regulates Important Immuno-metabolic pathways related to foam cell formation

Figure 45. is similar to Figure 40. but with the color-coded role of indole in Immuno-metabolic pathways that are important in the process of Mac-derived foam cell formation. Indole is regulating many pathways concerning inflammation, polarization, and metabolism of Macs.

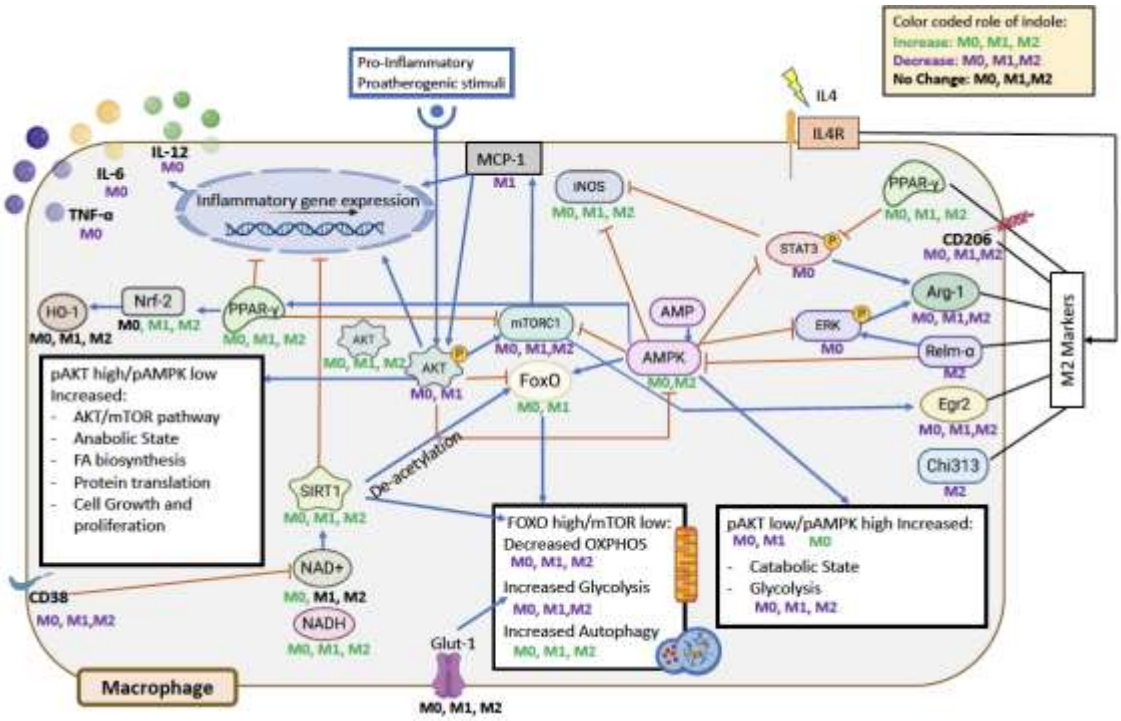


Figure 45. Indole regulates Important Immuno-metabolic pathways related to foam cell formation.

Immuno-metabolic pathways that are important in the process of Mac-derived foam cell formation. Color-coded role of indole in the regulation of each metabolic element. ATGL, adipose triglyceride lipase; PPAR, peroxisome proliferator-activated receptor; NAD, Nicotinamide adenine dinucleotide; NADH, Nicotinamide adenine dinucleotide + hydrogen (H). ; NADP, Nicotinamide adenine dinucleotide phosphate; NMN, nicotinamide mononucleotide, a competitor of NAD+ and alternative substrate of CD38; CD38, Cluster of differentiation 38, one of the main cellular NADases in mammalian tissues; SIRT1, Sitrulin 1, an enzyme that deacetylates proteins that contribute to cellular regulation; FOXO, Members of the class O of forkhead box transcription factors; MCP-1, The monocyte chemoattractant protein-1; iNOS, Inducible nitric oxide synthase; STAT3, signal transducer and activator of transcription 3; Arg-1, Arginase 1; Relm-α ,Resistin-like molecule alpha; Egr2, Early growth response protein 2; CD206, mannose receptor and C-type lectin; Chi313, chitinase-like lectin; ERK, extracellular signal-regulated kinase; AMP, adenosine monophosphate; AMPK, 5' AMP-activated protein kinase; mTORC1, mammalian target of

rapamycin complex 1; AKT, Protein kinase B (PKB); Nrf-2, nuclear factor erythroid-derived 2-like 2; HO-1, Heme oxygenase-1; Glut-1, Glucose transporter 1; TNF, Tumor necrosis factor; IL-12, Interleukin 12; IL-6, Interleukin 6, IL4R, The interleukin 4 receptor.

TMAO exacerbates the M1 polarization, and indole inhibits its effects

TMAO

As we have explained in chapter I, TMAO is a microbiota dependent metabolite that has a direct association with atherosclerosis. Patients with atherosclerosis have high plasma levels of TMAO and lower plasma levels of indole (238). That's why we became interested in checking the role of indole on M1 polarization of BMDMs, and to test this Day 7, BMDMs were pretreated with DMF solvent control, indole, TMAO, or indole + TMAO overnight, then we stimulated them with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) M2 (20ng/ml IL-4). BMDMs from WT mice, (2x 10^5 cells) stained extracellularly for CD11b and F4/80 as Mac markers and CD38 as M1 marker and then analyzed using FACS. Percentage of CD38-positive cells is indicated on the dot plots (Figure 46. A) and bar graphs (Figure 46. B). Our data indicate that TMAO highly increases M1 polarization of BMDMs, and when TMAO and indole are pretreated together, indole can significantly reduce the TMAO induced M1 polarization. These data suggest that indole activates pathways that inhibit the TMAO induced M1 polarization of Macs.

Mean fluorescence intensities (MFI) of the CD38-positive population in M1 TMAO were measured (Figure 46. C).

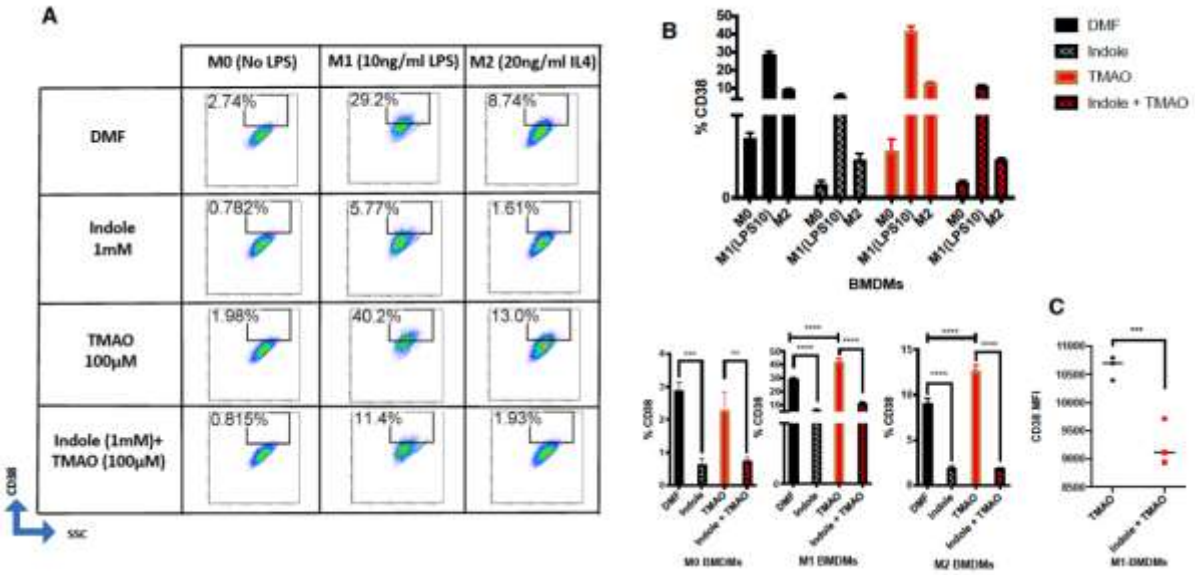


Figure 46. TMAO exacerbates the M1 polarization, and indole inhibits its effects.

Day 7 BMDMs were pretreated with DMF control, indole, TMAO, and Indole + TMAO overnight, then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) M2 (20ng/ml IL-4) (A) Flow cytometric analysis of BMDMs from WT mice, (2x 105) cells stained extracellularly for CD11b and F4/80 as Mac markers and CD38 as M1 marker using FACS. Percentage of CD38-positive cells are indicated on (A) dot plots and (B) bar graphs. (C) mean fluorescence intensities (MFI) of the CD38-positive population in M1 TMAO pretreated groups. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls performed using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$ or based one-way ANOVA with Tukey's multiple comparison test at "*" $p < 0.05$, significance at "**" $p < 0.01$, significance at "***" $p < 0.001$, significance at "****" $p < 0.0001$.

Indoxyl sulfate exacerbates the M1 polarization, and indole inhibits its effects

Indoxyl sulfate

As explained in chapter I, Indoxyl Sulfate (I3S or IS) is a microbiota related cardiotoxin and uremic toxin (35-40). In contrast to indole, indoxyl sulfate increases the inflammatory reactions in the cell and body (44). Studies have indicated that high levels of I3S are correlated with high risks of atherosclerosis (41,43). To check the role of I3S on M1 polarization of BMDMs and also in order to check the role of indole on I3S induced M1 polarization, day 7 BMDMs pretreated with DMF solvent control, indole, Indoxyl Sulfate, or Indole + Indoxyl Sulfate overnight and then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS), M2 (20ng/ml IL-4) BMDMs from WT mice, (2x 10^5 cells) stained extracellularly for CD11b and F4/80 as Mac markers and CD38 as M1 marker and then analyzed using FACS. The percentage of CD38-positive cells is indicated on the dot plots (Figure 47. A) and bar graphs (Figure 47. B). I3S increased the M1 polarization in BMDMs, and when I3S and indole pretreated together, indole inhibited the I3S induced M1 polarization. These data together suggest that indole can be a good endogenous candidate to reduce the M1 polarization of Macs and consequently reduce the foam cell formation.

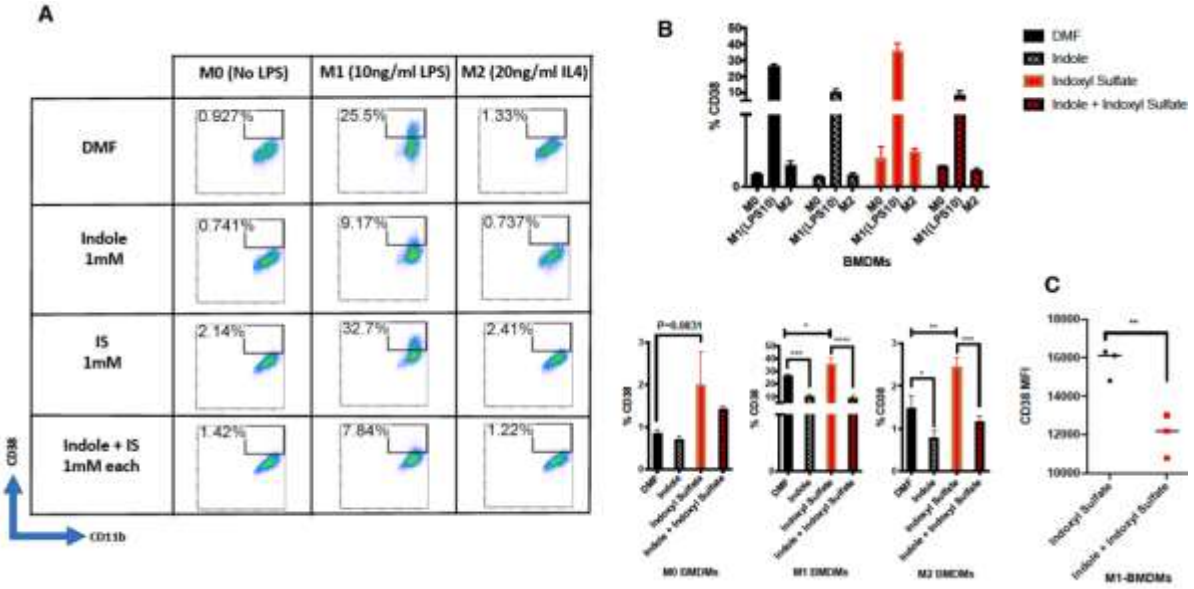


Figure 47. Indoxyl sulfate exacerbates the M1 polarization, and indole inhibits its effects.

Day 7BMDMs were pretreated with DMF control, indole, Indoxyl Sulfate, and Indole + Indoxyl Sulfate overnight, then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) M2 (20ng/ml IL-4) (A) Flow cytometric analysis of BMDMs from WT mice, (2x 105) cells stained extracellularly for CD11b and F4/80 as Mac markers and CD38 as M1 marker using FACS. Percentage of CD38-positive cells are indicated on (A) dot plots and (B) bar graphs. (C) mean fluorescence intensities (MFI) of the CD38-positive population in M1 indoxyl sulfate pretreated groups. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls performed using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$ or based one-way ANOVA with Tukey's multiple comparison test at "*" $p < 0.05$, significance at "**" $p < 0.01$, significance at "***" $p < 0.001$, significance at "****" $p < 0.0001$.

Discussion

Our group has determined that indole has profound immunomodulatory properties for regulating T-cells (data not shown) and APCs. Indole augments Treg differentiation via AhR (data

not shown); however, the effects of indole on Macs are AhR-independent; thus, indole regulates immune cells via the AhR and alternate pathways. Therefore, I tested my observations in WT vs. AhR-KO macs to determine AhR-dependent properties of indole for preventing foam cell formation. Next examined the effects of indole on Macs (BMDMs) in the context of saturated fatty acid-induced inflammation. My results demonstrated that the Indole pretreatment of BMDMs reduces palmitate-stimulated pro-atherogenic TNFα production. M1 polarization causes phenotypic and metabolic reprogramming of Macs that promotes atherosclerosis. I found that indole reduced the expression of several M1 polarization markers (CD38, MCP-1, glycolysis, Lactate production), suggesting that indole is capable of reducing M1 polarization.

Atherosclerotic plaque Macs resemble polarized M1 phenotype (high inflammation and glycolytic metabolism), whereas M2 Macs (low inflammation and oxidative phosphorylation metabolism) prevent plaque formation (161). To test the indole regulation of Mac's polarization, I analyzed in real-time metabolic parameters such as glycolysis and Oxidative phosphorylation (OXPHOS) changes in polarized BMDMs by examining extracellular acidification rate (ECAR), and the mitochondrial oxygen consumption rate (OCR) using Seahorse flux analyzer. As expected, M1 Macs had a high glycolytic capacity in response to LPS (212), and indole inhibited the glycolytic capacity of M1 Macs. My results suggest that Indole treatment reduced inflammatory, metabolic programming of M1 polarized Macs. However, we observed that indole reduces the expression of some M2 markers (CD206, Egr2, Arg-1) and increases some other M2 markers (PPAR γ, STAT3, AMPK). Our data suggest that indole does not switch the M1 polarization into M2 polarization. Instead, indole induces a unique phenotype in the Macs that is not 100% M1 or 100% M2, but we know that indole can definitely reduce M1 polarization of BMDMs even when

the cells are pretreated with TMAO or I3S. TMAO and I3S are microbiota dependent and derived metabolites that can exacerbate the progression of atherosclerosis (28, 41).

My data has shown that TMAO and I3S can significantly increase the M1 polarization, and indole can inhibit their induced M1 polarization in BMDMs. These findings are particularly novel. In this chapter, we demonstrated that indole regulates some metabolic pathways that are involved in the process of foam cell formation. Generally, when the Macs are in inflammatory states, they are more prone to transform into foam cells (116), and our lab's previous data and my data has shown that indole can significantly reduce the inflammatory cytokines. The effects of indole on the regulation of metabolic pathways that I have measured so far were independent of AhR. Interestingly, in contrast to T-cells, and based on our previous lab data, the regulatory role of indole in dendritic cells was also independent of AhR. These suggest that role of AhR on Macs and DCs is probably regulated differently based on the origin of the AhR ligand. Our results have shown that indole's suppressive effects in APCs, is not due to inhibition of APC maturation. And this suggests that there are other factors and pathways involved in the indole's mechanism of actions.

In this chapter, we demonstrated a novel modulation of Macs via a small endogenous microbiota-derived metabolite. Macs /foam cell metabolism has a critical role in the pathogenesis of atherosclerosis (116), and since diet and microbiota have a direct link with atherosclerosis (177, 179), we measured the role of indole on some of the important metabolic pathways in the Macs.

Our previous lab data has indicated that indole induces bacterial killing properties in the cell. My data indicated that indole increases the iNOS and ROS production in the Macs, which we hypothesis that this may be due to indole’s bacterial killing induction.

Our data suggested that indole pretreatment increases the expression on NADH and NAD. This suggests that indole may upregulate the pathways that are dependent on NAD+/ NADH.

For example, we measured the expression of SIRT1 that is dependent on NAD+/NADH pathway (239), and our data indicated that indole increases SIRT1 and since NAD+/ SIRT pathway can regulate the cell metabolism by activating FOXO signaling (240), we measured the expression of FOXO and noticed that indole increases FOXO1 expression in M0 and M1 Macs. Interaction of FOXO and SIRT1 has been associated with the regulation of pathways that are involved in atherosclerosis and aging metabolism (229). Published data suggest that inhibition of NAD+/SIRT1 pathways can lead to the reduction of FOXO and consequently, exacerbation of atherosclerosis (240). Indole can be a good endogenous candidate to induce the NAD+/SIRT1/FOXO1 pathway. Furthermore, my data indicated that indole increases the Nrf2 expression in polarized Macs. Nrf-2 expression regulates inflammation and has a role in the downregulation of free radicals. Free radicals are the main cause of oxidation of LDLs in the body, and ox-LDLs are the main proatherogenic particles in atherosclerosis (241).

However, indole did not change the expression of HO-1, which is a Nrf2-regulated gene that has an essential role in the prevention of vascular inflammation (233). Since Nrf2 is upregulated by PPAR- γ (235), we measure the expression of PPAR- γ as well and noticed that indole increases PPAR- γ expression in M0, M1, and M2 BMDMs. PPAR- γ also has inhibitory effects on inflammatory cytokines expression, and it is an M2 marker (208, 242).

Our data indicated that indole decreases glycolysis and OXPHOS in a media that contains glucose and fatty acid is not present in the media as the main fuel. On the other hand, published data suggest that FOXO1 may have a role in the induction of switching carbohydrate energy production to the fatty acid oxidation pathway as the main energy-producing path (232).

Future investigation into the details of each metabolic pathway regulated by indole and the binding site of indole (which is probably not AhR) will be extremely important in further understanding the biological activities of indole in the Macs.

Together, our data suggest that indole is probably activating other cell pathways to compensate Mac's energy demands. That's why in the next chapter, we will discuss the role of indole on Fatty acid oxidation foam cell formation.

CHAPTER 3

MECHANISMS USED BY THE MICROBIOTA AND INDOLE TO REGULATE FOAM CELL FORMATION IN RESPONSE TO PRO-ATHEROGENIC LIPOTOXIC STIMULI.

Overview

A key feature of atherosclerotic plaques is the formation of foam cells caused by lipid homeostasis and Macs' degradation. Lipid accumulation in foam cells initiates atherosclerotic plaque formation in the aorta, and when foam cells are reduced/inhibited atherosclerosis is reduced/inhibited. Mechanisms that minimize Macs inflammation, increase lipid degradation, and prevent foam cell formation, are likely to decrease atherosclerosis progression. We hypothesized that indole prevents foam cell formation in response to oxLDL. My objective was to study the mechanisms and pathways whereby indole prevents foam cell formation. Mouse models of high-fat diets have been used to demonstrate a link between microbiota composition and the development of obesity, diabetes, and atherosclerosis through several mechanisms such as TMAO and SCFAs. This association between obesity, inflammation, and the microbiota has led me to formulate the hypothesis that additional microbiota-derived metabolites in the GI tract (i.e., Trp metabolites) enter circulation and regulate dietary saturated fatty acid-induced phagocyte inflammation. I have tested the role of indole and the detrimental TMAO on foam cell formation in vitro. There are no direct studies on the effects of indole and TMAO in foam cell formation.

The following findings emerged:

1. Indole inhibits oxLDL induced foam cell formation in Macs.
2. Indole regulates foam cell formation by reducing the fatty acid synthesis and increasing FA oxidation.
3. Indole induction of lipid catabolism and inhibition of lipid synthesis in the Macs is independent of AhR.

Rationale

Many pieces of research all around the world have indicated the important link between gut microbiota and CVD, particularly atherosclerosis (20). Atherosclerosis is the leading cause of death in western countries (242), suggesting that the prevalence of atherosclerosis has other reasons beyond the traditional risk factors. Patients diagnosed with atherosclerosis indicate gut dysbiosis signs (243). And dysbiosis is one of the pathological manifestations of atherosclerosis (243). TMAO is one of the fairly studied gut microbiota dependent metabolites and has a direct correlation with the progression of atherosclerosis (28). Most of the studies regarding microbiome metabolites and CVD have been on serum levels of a specific metabolite in the patient or the mouse model (244). The direct role microbiota metabolites on the Macs or foam cells have been poorly addressed. Since the Macs and especially lipid-laden Macs or foam cells are the primary cells, accumulated in the arteries (245), our main focus is the role of gut microbiota metabolites, especially indole in the regulation of the Macs and foam cells. In the previous chapter, we demonstrated the role of indole in the regulation of the inflammation of the Macs. In this chapter, other than inflammation, we will focus on the other proatherogenic signals that can induce foam cell formation of Macs and investigate the role of indole in regulating these factors.

Studies have shown that a high-fat diet is one of the proatherogenic factors suggesting the detrimental role of excess lipids and fatty acids in inducing proatherogenic inflammatory states in the body (246). We have tested the role of indole in high-fat-diet-induced Macs. Saturated fatty acids are the other pro-atherogenic factors that can directly affect the formation of inflammatory Macs and foam cells. That's why we have tested the role of indole in the regulation of fatty acid-induced inflammation.

On the other hand, it has been reported that the inflammatory Macs and foam cells have altered metabolic states compared to naïve or anti-inflammatory Macs (247). To this end, we tested the metabolic pathways that have important roles in the foam cell formation of Macs, and we investigated the role of indole in the regulation of these pathways.

Finally, we set out to determine whether the roles of indole in the regulation of foam cell formation is dependent on the AhR or not.

Results

Indole inhibits the foam cell formation, and in indole with TMAO conditions, indole is dominant at inhibiting the foam cell formation

Lipid accumulation in foam cells initiates atherosclerotic plaque formation in the aorta, and when foam cells are reduced/inhibited atherosclerosis is reduced/inhibited (30). Studies on the direct effects of TMAO on foam cell formation are limited. We tested the role of indole and the detrimental pro-atherogenic TMAO on foam cell formation in vitro. Day 7 BMDMs were starved in 1% FBS RPMI-c, pretreated with DMSO, Rapamycin, TMAO, Indole, and 3MA. Then, the cells were then incubated with LDL or oxLDL for 24h before being fixed with 4% PFA and stained with Oil Red O.

My data demonstrated that TMAO enhances the foam cell formation while indole inhibits foam cell formation. Furthermore, in indole + TMAO conditions, indole is dominant at inhibiting foam cell formation (Figure 48).

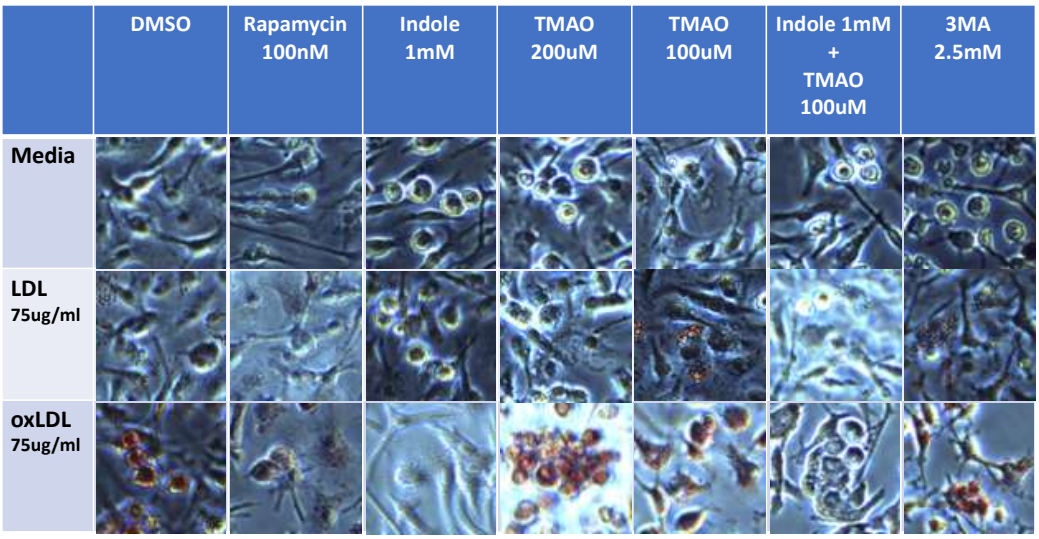


Figure 48. Indole inhibits the foam cell formation, and in indole with TMAO conditions, indole is dominant at inhibiting the foam cell formation.

Day 7 BMDMs were starved in 1% FBS RPMI-c, pretreated with DMSO, Rapamycin, TMAO, Indole, and 3MA. The cells were then incubated with LDL or oxLDL for 24h before being fixed with PFA and stained with Oil Red O.

Indole increases FAS gene expression in LPS induce M1 BMDMs independent of AhR

FAS (Fatty acid synthase) is an enzyme that its function is to catalyze fatty acids, especially the synthesis of palmitate from acetyl-CoA and malonyl-CoA (248). Since we observed the indole-induced reduction of foam cell formation in BMDMs, we became interested in testing the role of indole in FAS regulation. To perform this experiment, we used day 7 BMDMs from WT or AhR-KO mice and pretreated the cells with indole or DMF solvent control overnight. Then we stimulated the cells with polarization skews for 24 hours as follows: M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS), and M2 (20ng/ml IL-4). Relative gene expression of FAS, measured in polarized BMDMs using qRT-PCR. Our results indicated that M1 Macs highly express FAS, and indole significantly reduced the expression of FAS in M1 BMDMs. The activity of Indole was AhR independent (Figure 49). Our data suggest that reduction in the expression of FAS may be one of the mechanisms of action of indole in the reduction of foam cell formation.

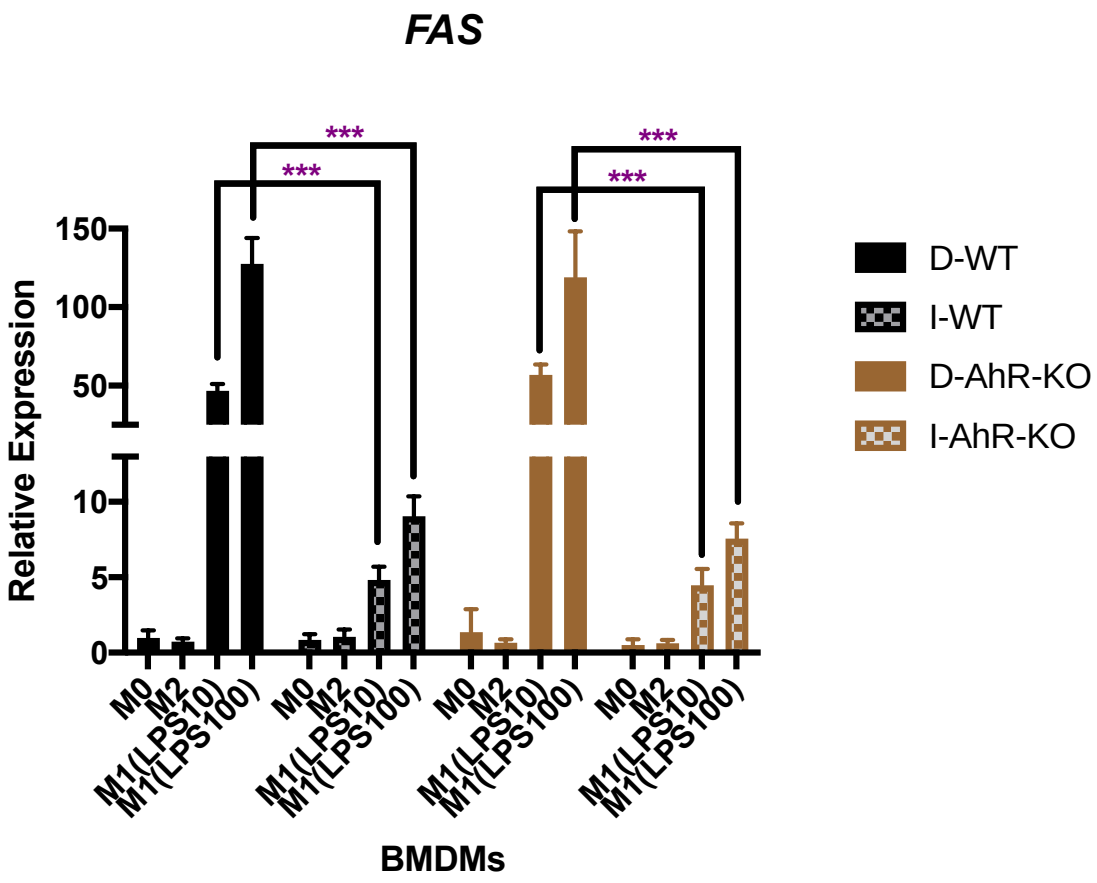


Figure 49. Indole increases FAS gene expression in LPS induce M1 BMDMs independent of AhR.

Day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of FAS, measured in polarized BMDMs using qRT-PCR. FAS, Fatty acid synthase. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Indole slightly increases gene expression in BMDMs, independent of AhR

Increased inflammation and inhibited cholesterol efflux lead to the accumulation of cholesterol and triglycerides and increase the lipid droplets and foam cell formation in proinflammatory Macs (249). ABCG1 (ATP-binding cassette sub-family G) belongs to ABC transporters subfamily and has a role in cholesterol efflux and lipid-trafficking regulations in Macs (249). Based on foam cell formation results, we hypothesized that indole could increase ABCG1 activity and increase cholesterol efflux in BMDMs. To test the expression of ABCG1, we used day 7 BMDMs from WT or AhR-KO mice and pretreated the cells with indole or DMF solvent control overnight. Then we stimulated the cells with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of ABCG1, measured in polarized BMDMs using qRT-PCR. Our results indicated that indole slightly increases the expression of ABCG1 in BMDMs independent of AhR (Figure 50). The indole induced increase in the ABCG1 gene in BMDMs was very little. That's why we tested the role of indole on lipolysis and fatty acid oxidation to have a better understanding of the role of indole in the regulation of lipid droplets.

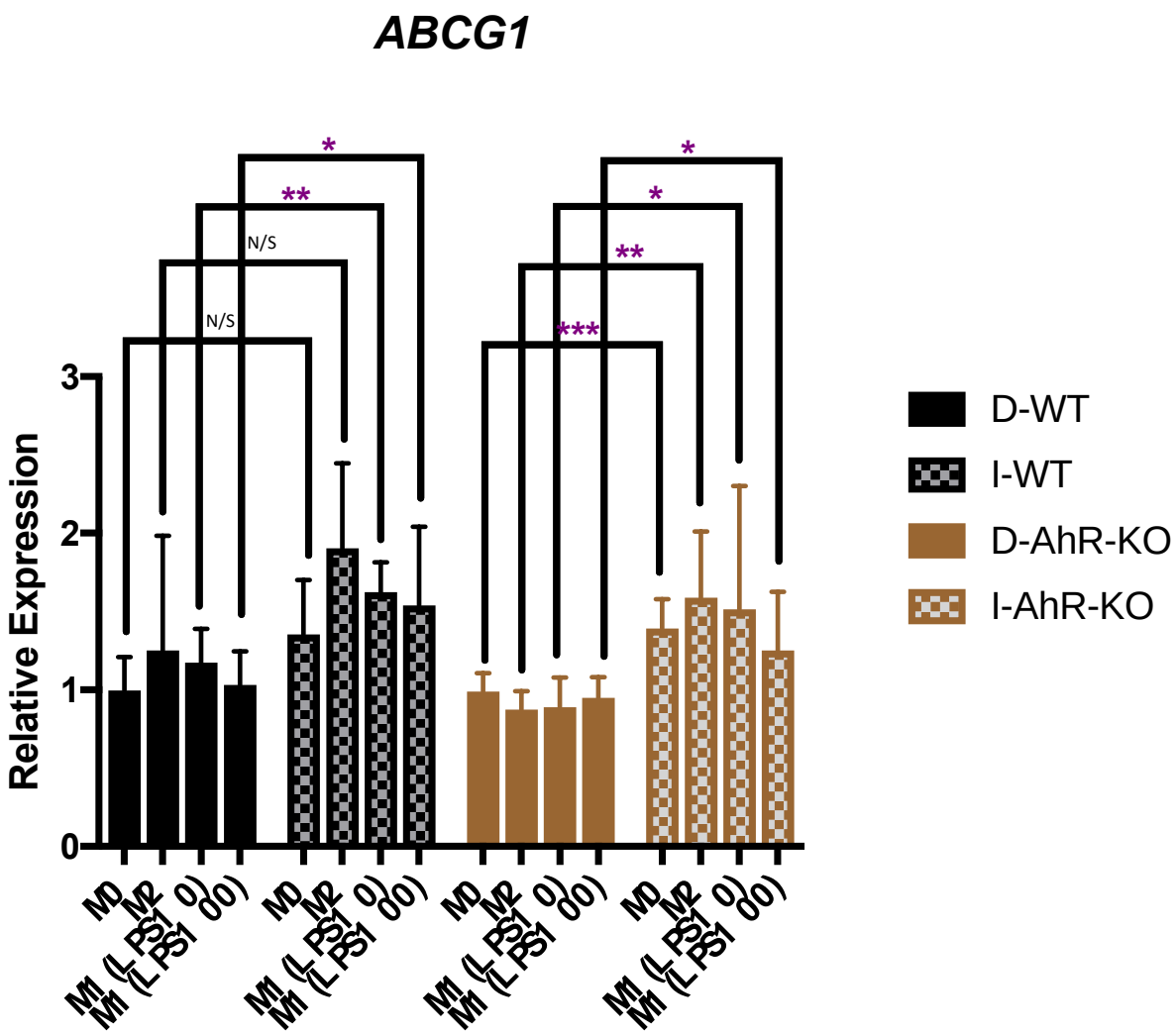


Figure 50. Indole slightly increases ABCG1 gene expression in BMDMs, independent of AhR.

Day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of ABCG1, measured in polarized BMDMs using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Indole increases ATGL gene expression in BMDMs independent of AhR

ATGL (Adipose triglyceride lipase) has a role in the catabolism of the fats and hydrolysis of triglyceride (250). Based on our foam cell formation results, we hypothesized that indole could increase ATGL activity to decrease the stored fat in BMDMs. To test this, we used day 7 BMDMs from WT or AhR-KO mice. The cells were pretreated with indole or DMF solvent control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of ATGL, measured in polarized BMDMs using qRT-PCR. Our results indicated that indole increases the ATGL expression in M0, M1, and M2 BMDMs.

Our results indicated that indole increases ATGL expression (Figure 51). One of the reasons for indole-induced reduction of foam cell formation is that indole is increasing ATGL, which leads to the breaking up of the lipid droplets. This suggests that there will be more free FA in the cell, and the FFA can be utilized to produce ATP through the enhanced FAO pathway. We have tested the FAO and CPT-1 in the next section. The regulatory roles of indole in lipid metabolism were independent of AhR.

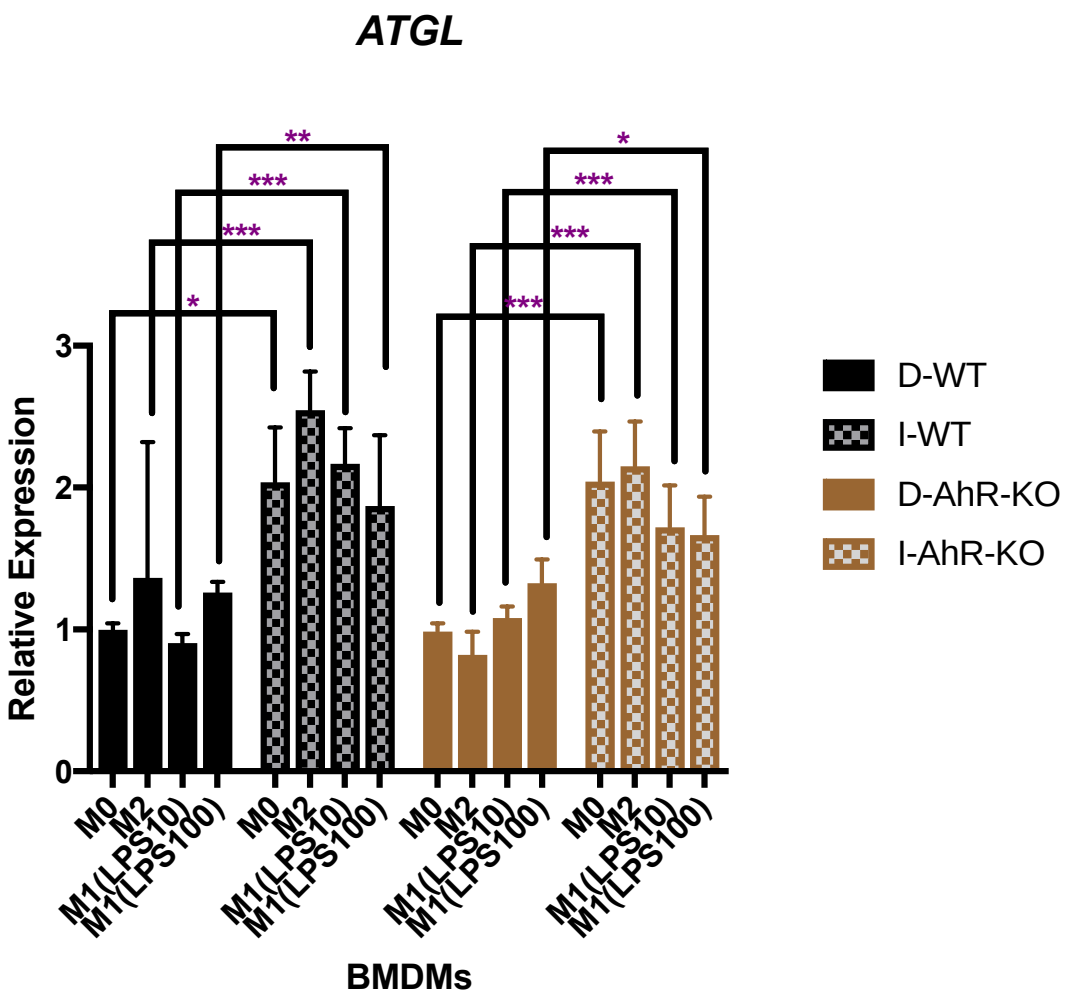


Figure 51. Indole increases ATGL gene expression in BMDMs independent of AhR.

Day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of ATGL, measured in polarized BMDMs using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Indole increases CPT-1 gene expression in BMDMs independent of AhR and increases FAO in BMDMs

CPT-1 (Carnitine palmitoyl transferase 1), is an Enzyme in the mitochondria that has an essential role in the beta-oxidation of long-chain fatty acids. Defections in CPT-1 prevents the body from using fats to produce ATP (251). To check the role of indole of the regulation of CPT-1 expression, we used day 7 BMDMs from WT or AhR-KO mice and we pretreated them with indole or DMF solvent control overnight then stimulated them with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of ATGL, measured in polarized BMDMs using qRT-PCR. Our results indicated that indole increases the CPT-1 expression in all of our polarized BMDMs (Figure 52. A). This pathway may have a small change but the effects of indole seems to be across multiple pathways so adding these up leads to strong function of inhibition of foam cells.

In Fatty acid oxidation (FAO) or beta-oxidation, fatty acids break down in the cell's mitochondria to produce acetyl-CoA, which will enter into the TCA cycle and eventually lead to the production of ATP through mitochondrial electron transport chain (251). We perform the Seahorse Fatty acid oxidation test to measure the FAO of the BMDMs in real-time. We used day 7 BMDMs and pretreated the cells with indole or DMF solvent control overnight. Then we performed FAO test based on the manufactures protocol. Fatty acid oxidation of Day 7 BMDMs indicated as OCR (pmol/min). our data indicated that indole increases the fatty acid oxidation in the BMDMs (Figure 52. B). These data were in line with our previous data regarding indole-induced reduction of foam cell formation.

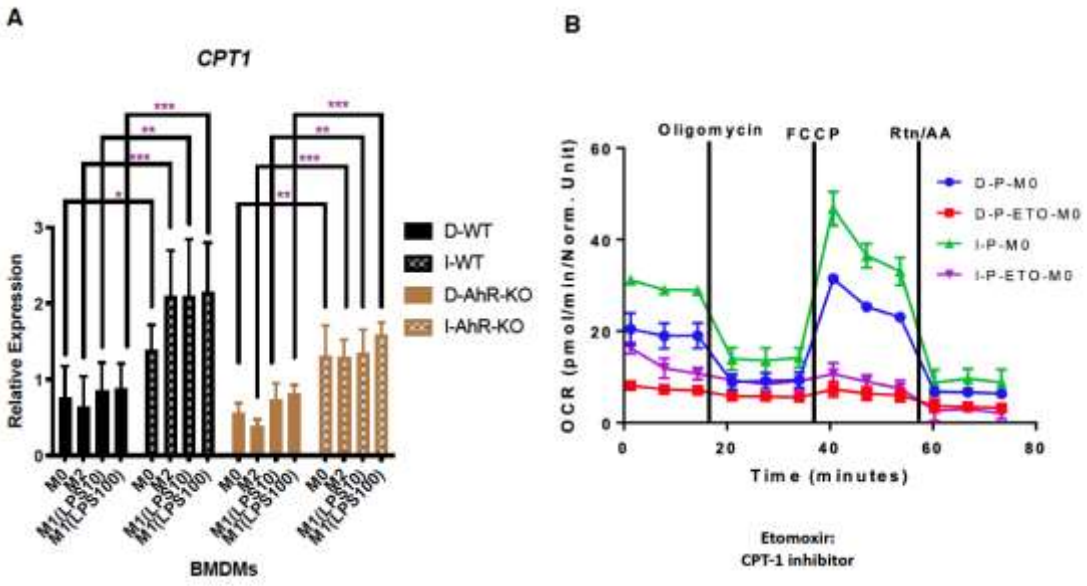


Figure 52. Indole increases CPT-1 gene expression in BMDMs independent of AhR and increases FAO in BMDMs.

(A) Day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of CPT-1 measured in polarized BMDMs using qRT-PCR. (B) Day 7 BMDMs were pretreated with indole or DMF solvent control overnight. Fatty acid oxidation of Day 7 BMDMs indicated as OCR (pmol/min). Basal Respiration, ATP Production, Proton Leak, Maximal Respiration, and Spare Respiratory Capacity as OCR (mpH/min) are the same as the Mito-stress test A: Oligomycin, Port B: FCCP, Port C: Rotenone plus antimycin A and. Data shown are from at least four culture wells per experiment. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Indole does not alter the expression of CD36 (scavenger receptor class B).

CD36 (AKA, fatty acid translocase, glycoprotein 88, platelet glycoprotein 4 or scavenger receptor class B member 3) is a membrane protein expressed in many types of mammalian cells (252). Scavenger receptors are known for their tendency to recognize and bind to various ligands such as lipoproteins, cholesterol esters, phospholipids, carbohydrates, and polyanionic ligands. That is the reason why the scavenger receptors have a significant role in the homeostasis of the cells (253). Scavenger receptors also have roles in the control of inflammatory diseases. They can recognize PAMPs and DAMPs and scavenge the cells from pathogens, ROS, and apoptotic cells (254). Inflammatory Macs in atherosclerosis highly express scavenger receptors to take up ox-LDL (253, 254). Furthermore, the inflammatory Macs that highly express scavenger receptors secrete more inflammatory cytokines and exacerbate plaque formation (30).

Since we observed the broad role of indole in the regulation of foam cell formation, we became interested in testing the role of indole in the expression of CD36 as one of the scavenger receptors. We used day 7 BMDMs and pretreated them with DMF solvent control, indole, Indoxyl Sulfate, and Indole + Indoxyl Sulfate overnight, then stimulated them with polarization skews 24 hours. M0 (Media), M1 (10ng/ml LPS) M2 (20ng/ml IL-4). Then we stained the cells for CD11b and F4/80 to recognize Macs and CD36 to measure the scavenger receptor expression. In contrast to our hypothesis, indole did not change the expression of CD36 (Figure 53). Our data is suggesting that indole is not regulating CD36 to reduce foam cell formation. How ever, we have not tested

other scavenger receptors. Investigating the role of indole in the regulation of other scavenger receptors in Macs is highly suggested.

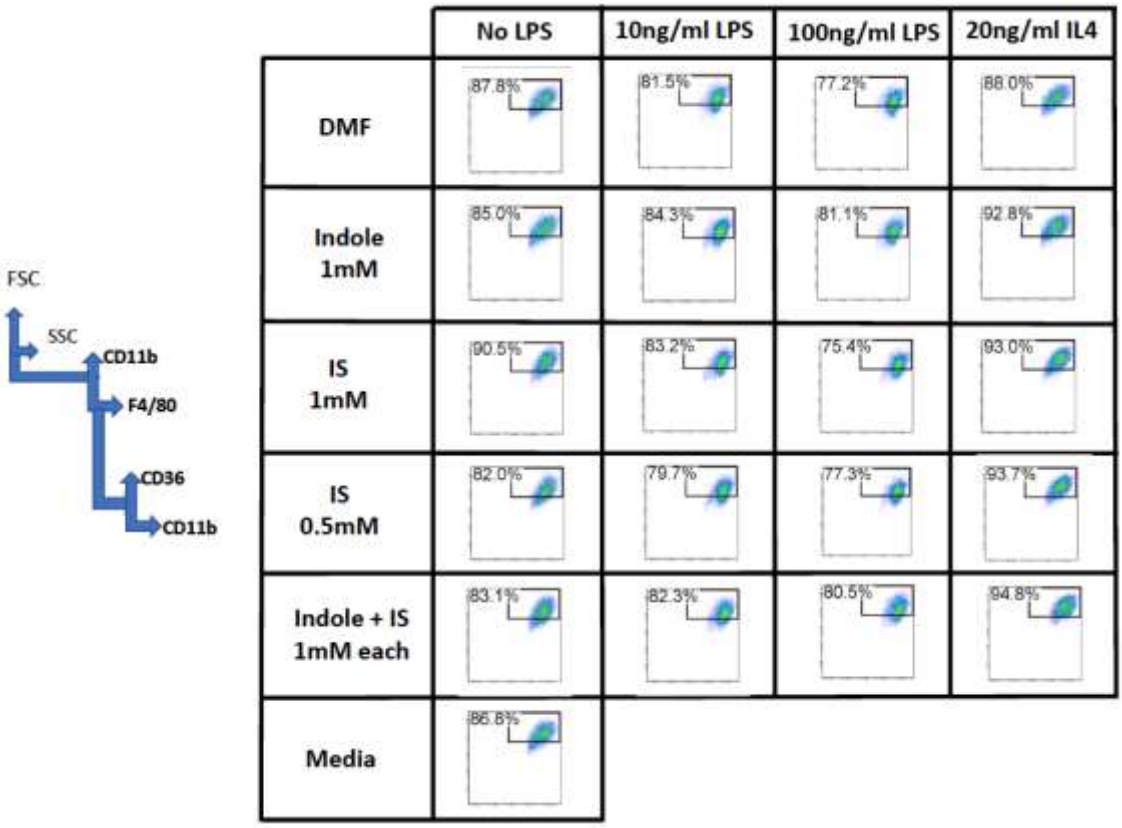


Figure 53. Indole does not alter the expression of CD36 (scavenger receptor class B).

Day 7 BMDMs were pretreated with DMF control, indole, Indoxyl Sulfate, and Indole + Indoxyl Sulfate overnight, then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) M2 (20ng/ml IL-4). Flow cytometric analysis of BMDMs from WT mice. (2x 10^5) cells stained extracellularly for CD11b and F4/80 as Mac markers and CD36 using FACS. Percentage of CD36-positive cells indicated on dot plots. Data are representative of one of three experiments.

Indole increases AMPK gene expression in BMDMs independent of AhR

Published data so far has indicated that AMPK increases cholesterol efflux, activates autophagy, reduces inflammation, and attenuates atherosclerosis. Since AMPK and mTOR regulate metabolism, which is essential in lipid-metabolism related to atherosclerosis (255). We tested indole regulation of AMPK expression in BMDMs.

To test this, we used day 7 BMDMs from WT or AhR-KO mice and pretreated them with indole or DMF solvent control overnight then stimulated them with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of AMPK measured in polarized BMDMs using qRT-PCR.

Our results indicated that indole slightly increases AMPK expression in M0 and M2 BMDMs (Figure 54). However, indole doesn't change the expression of AMPK in M1 BMDMs. The indole regulation of AMPK was independent of AhR. Furthermore, our previous lab data has indicated that indole significantly increases the AMPK activation (data not shown).

Our overall data suggest that indole upregulates the AMPK regulation, and since the effects of indole in Macs are AhR independent, we hypothesize that indole has a role in the regulation of autophagy. In the next chapter, we will discuss more regarding the role of indole in the regulation of autophagy.

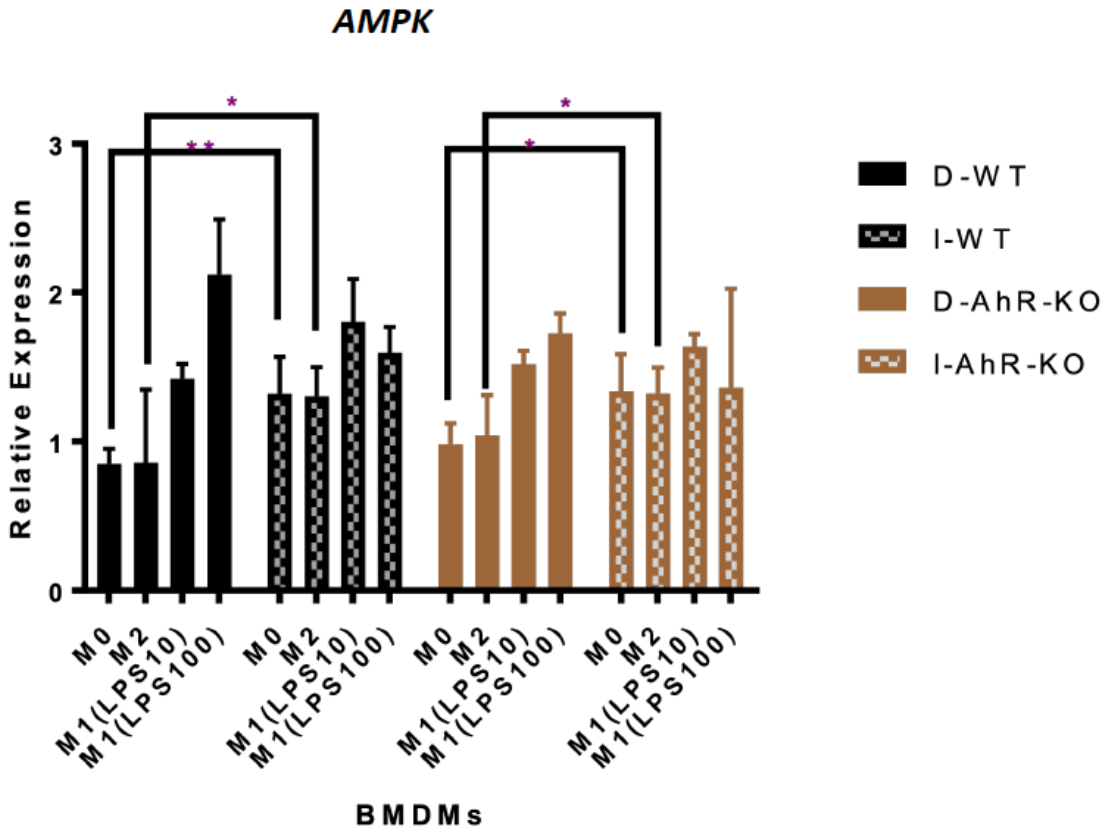


Figure 54. Indole increases AMPK gene expression in BMDMs independent of AhR.

Day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of AMPK measured in polarized BMDMs using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Our data with regard to lipid metabolism has indicated that indole can regulate various pathways to control and reduce lipid accumulation and foam cell formation. Table 2 is a color-coded list of the genes, proteins, and cell functions in the Macs that we have tested (AMPK protein

information is from previous lab data) the role of indole on their regulation. We have also mentioned the indole's regulation dependency on AhR.

Table 2. Table of indole's effects on Mac gene, protein, function, and dependency on AhR in lipid-metabolism related factors.

Lipid-metabolism

Color coded role of indole:
Increase: M0, M1, M2
Decrease: M0, M1,M2
No Change: M0, M1,M2

Immuno-metabolism	Gene	Protein	Function	Dependency on AhR
FAO Oxidation			M0, M1, M2	
ABCG1	M0, M1, M2			Independent
CPT-1	M0, M1, M2			Independent
FAS	M0, M1, M2			Independent
ATGL	M0, M1, M2			Independent
CD36		M0, M1, M2		Independent
Foam cell formation			M0, M1	
PPAR-γ	M0, M1, M2			Independent
SIRT-1				Independent
IL-12		M0		Independent
TNF-α		splenic Macs		Independent
		Palmitate stimulated M0		
		splenic M2		Independent
IL-6		M0		
AMPK	M0, M2	M0		Independent

Indole regulates Important lipid metabolism pathways related to foam cell formation

The information in Figure 55. is similar to Table 2. but with the color-coded role of indole in Lipid-metabolism pathways that are important in the process of Mac-derived foam cell formation. In the previous chapter, our data suggested the anti-inflammatory roles of indole. Generally, increased levels of inflammation lead to increased levels of lipid accumulation in Macs (4). In chapter 1, we indicated that indole has anti-inflammatory roles in Macs. Our data showed that indole significantly inhibits the foam cell formation of Macs. It increases the expression of the

lipolysis gene (ATGL), increases FAO, and significantly reduces the FAS enzyme expression. Indole increases the expression of the ABCG1 gene, which has a role in cholesterol efflux. Indole increases the expression of AMPK and SIRT-1 genes. Both of these genes have inhibitory effects on lipid synthesis. Although published data propose that PPAR- γ may be pro-lipid synthesis (249, 255), but on the other hand, its anti-inflammatory effects have been dominant on its pro-lipid-synthesis effects (241). Indole increases the expression of CPT-1 enzyme, which is an essential enzyme in beta-oxidation of fatty acids (251).

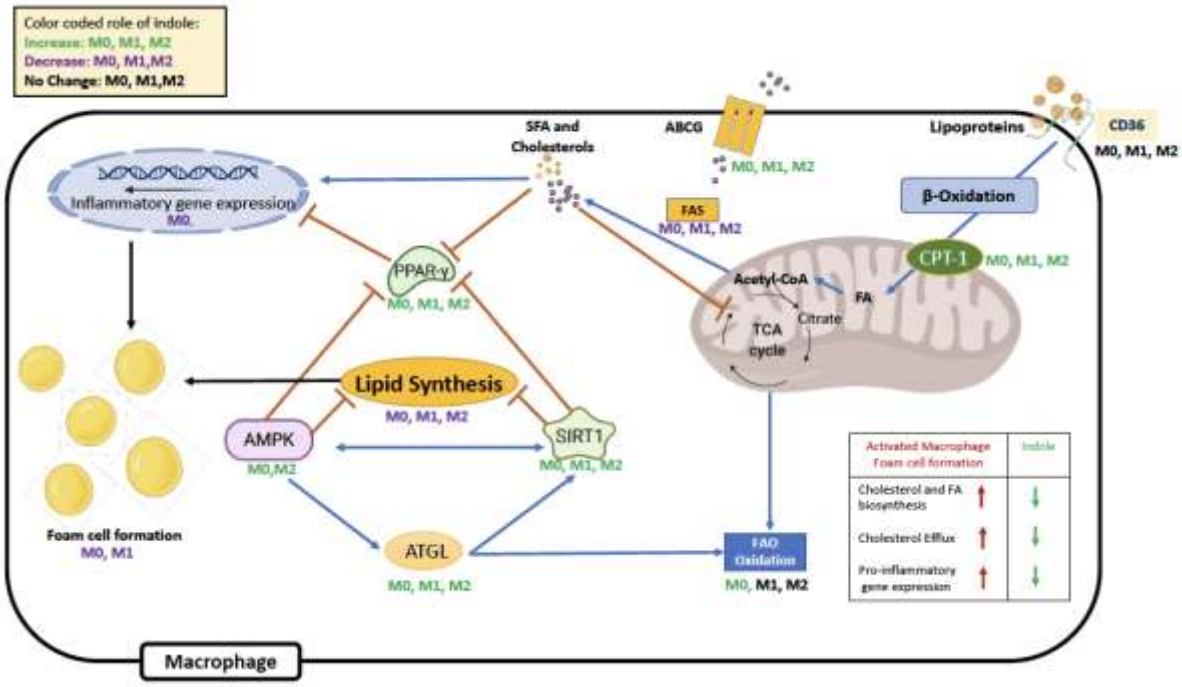


Figure 55. Indole regulates Important lipid metabolism pathways related to foam cell formation. Lipid-metabolism pathways are essential in the process of Mac-derived foam cell formation—the color-coded role of indole in the regulation of each metabolic element. ATGL, adipose triglyceride lipase; PPAR, peroxisome proliferator-activated receptor; SIRT1, Sitrulin 1, an enzyme that deacetylates proteins that contribute to cellular regulation; FOXO, Members of the class O of forkhead box transcription factors; AMPK, 5' AMP-activated protein kinase; SFA, Saturated fatty acid; FA, Fatty acid; FAS, Fatty acid synthase; ABCG, ATP-binding cassette sub-family G member; CD36, (cluster of differentiation 36), also known as scavenger receptor class B;CPT-1,Carnitine palmitoyl transferase 1.

Discussion

In chapter 3, we have investigated the role of (gut microbiota-derived) indole conditioning in Macs. And we tested its role in modulating lipid droplets accumulation and foam cell formation. Studies regarding the role of gut microbiota on Macs in atherosclerosis disease mode are minimal. Our data indicated that indole has inhibitory effects on foam cell formation in BMDMs. On the other hand, TMAO, which is a detrimental Microbiota dependent metabolite (24), increased foam cell formation. Interestingly, when we pretreated the TMAO and indole together, the indole effects were dominant, and foam cell formation was limited.

To understand the mechanisms behind the indole-inhibition of foam cell formation, we performed a set of experiments related to lipid metabolism in the Macs.

First, we tested the role of indole in the regulation of fatty acid synthesis. Fatty acid synthase is an essential enzyme in the process of fatty acids creation (248). Our data and other published has indicated that M1 Macs highly express the FAS enzyme (97). M1 Macs are the most populated type of Macs in atherosclerotic plaques (128). Indole pretreatment of the polarized BMDMs significantly reduced the expression of FAS in M1 Macs, both in WT and AhR-KO cells.

On the other hand, other mechanisms involved in the inhibition of foam cell formation could be the activation of catabolic pathways of fatty acids and lipids (96,97). We tested the expression of CPT-1 that is the rate-limiting enzyme in the entry process of long-chain fatty acids to the mitochondria and eventually, beta-oxidation (148). Indole pretreatment slightly but significantly increased the expression of CPT-1 in M0, M1, and M2 BMDMs and. Its effects were AhR independent. To test the fatty acid oxidation of indole pretreatment in BMDMs, we tested the

fatty acid oxidation of BMDM with and without indole pretreatment. We noticed that indole increases the FAO in real-time.

Since in chapter 2, we noticed that indole pretreatment decreases glycolysis and OXPHOS pathway in cells that were cultured in glucose-containing media, we hypothesized that indole is inducing fatty acid oxidation to meet the cells energy demand. Furthermore, in chapter 2, our data indicated that indole increases the expression of FOXO. A few data suggest that increased FOXO levels may have a role in switching the glycolytic energy production pathway to FAO (232). However, the mechanism behind this needs more investigation and broad, detailed measurements. The cell needs free fatty acids to activate beta-oxidation; This means that cells need to activate lipolysis metabolic pathway. In lipolysis, lipid triglycerides get hydrolyzed into glycerol and fatty acids. In this process, stored lipid droplets in the cytoplasm are hydrolyzed (148,149). Adipose triglyceride lipase (ATGL) enzyme is the rate-limiting enzyme in lipolysis. We measured the ATGL in indole or control pretreated BMDMs from WT and AhR-KO mice. Indole (slightly, but statistically significant) increased the expression of ATGL in M0, M1, and M2 BMDMs independent of AhR; This is another mechanism that can be involved in indole inhibition of foam cell formation.

Mac cholesterol efflux is one of the pathways that can control the accumulation of cholesterol in the cell. ATP-binding cassette transporters such as ABCG1 can modulate cholesterol efflux in Macs and foam cells. ABCG1 also regulates the inflammatory cytokines and chemokine responses of Macs (250). However, the mechanism behind this needs more investigation and broad, detailed measurements.

We checked the role of indole in the regulation of ABCG-1, and our results indicated that indole (slightly but statistically significant) increased the expression of ABCG1 in all types of our

polarized BMDMs. And this suggests another pathway of indole regulated-inhibition of foam cell formation.

We also tested one of the scavenger receptors in Macs. Indole did not change the expression of CD36 in the Macs. Testing other scavenger receptors is highly suggested.

So far, in my experiments, almost all of the regulatory effects of indole in Macs were AhR independent; This suggests that indole is probably regulating the lipid-metabolism and immune-metabolism with activating other pathways. Indole is a small infusible molecule and can regulate multiple pathways (50). Our previous lab data regarding the effects of indole on dendritic cells were also showing AhR independent role of indole. The receptor of indole in the regulation of Macs and dendritic cells is still unknown.

It is important to mention that rapamycin pretreatment, similar to indole pretreatment, significantly reduced the foam cell formation. Rapamycin is a potent inducer of autophagy, and 3MA is an inhibitor of autophagy (75-79). Our results indicated that 3MA exacerbates foam cell formation. On the other hand, indole increased AMPK expression in BMDMs and increased AMPK phosphorylation. AMPK activation leads to inhibition of mTOR and activation of autophagy (256).

These data led us to the hypothesis that activation of autophagy and lipophagy through indole pretreatment can be one of the mechanisms in indole-induced inhibition of foam cell formation.

Regulation of the pathways mentioned above in the Macs may induce a small change in the cell, individually, but the effects of indole seem to be across multiple pathways. So, adding these up, the indole-induced regulation of these pathways leads to a strong function of inhibition

of foam cells. Generally, indole regulates the Mac through a two-sided pathway. One side through lipid catabolism and on the other side by dramatically reducing fatty acid's ability to be generated.

The data in this chapter indicate that indole is using multiple mechanisms to inhibit foam cell formation.

CHAPTER 4

INDOLE INDUCED AUTOPHAGY REGULATES PHAGOCYTE RESPONSE TO PRO-ATHEROGENIC STIMULI.

Overview

Recycling program autophagy is critical to prevent lipo-toxicity and reduce foam cell formation in atherosclerotic Mac, and increased autophagy helps regulate lipid metabolism and prevent the development of atherosclerosis. Mechanisms that regulate autophagy in Macs will likely reveal targets for the prevention of atherosclerosis. Indole decreases innate and adaptive inflammation, promotes antimicrobial activities, and induces autophagy similar to rapamycin but without toxicity. We hypothesized that indole controls Mac inflammation/polarization and autophagy to prevent foam cell formation in response to ox-LDL and FAs. Our objective was to study the mechanisms and pathways whereby indole prevents foam cell formation and regulates Mac autophagy in response to atherogenic stimuli.

The following findings emerged:

1. Indole induces autophagy in Macs.
2. Indole-conditioned autophagy inhibits ox-LDL-induced lipid droplet formation in Macs.
3. Indole induction of autophagy is independent of AhR.

Rationale

Mac autophagy is an important cellular mechanism for optimum antigen/particulate degradation and reduced cellular inflammation (90). Most Macs in atherosclerotic plaques are filled with cytosolic lipid droplets (LDs) (30). In advanced atherosclerosis, Macs provide inflammatory signals and fail to effectively clear lipids and debris. Recently articles have established that phagocyte autophagy, when strongly active, is a critical cellular process that serves to prevent and eliminate plaque buildup in animal models of atherosclerosis (5,6). In addition, autophagy, has known to be critical in atherosclerotic plaque stability and Mac metabolism whereby increased autophagy decreases pro-inflammatory glycolytic metabolism (156).

An important biomarker of canonical autophagy is the conversion of microtubule-associated protein 1 light chain 3 (LC3-I) to a lipidated form LC3-II, which localizes to autophagic membranes (76). Importantly, the amount of LC3-II within a cell reflects the amount of newly formed autophagic membrane and/or the number of canonical autophagosomes (257).

In our preliminary lab data, consistent with the induction of autophagy, indole-conditioned APCs exhibited increased LC3-II expression and puncta formation, enhanced bacterial killing, and reduced inflammasome activity. Indole effects were largely similar to rapamycin yet also distinct, suggesting a complementary mechanism of action for these molecules. Both indole and rapamycin suppressed mTOR signaling and IL-1β production, suggesting a functional link. Unlike rapamycin, we have reported indole is a ligand of the aryl hydrocarbon receptor (AhR), yet regulation of autophagy by indole was AhR-independent. Autophagy plays an important role in immune defense as well. We hypothesize that indole is a major microbiota-derived regulator of phagocyte autophagy and inflammation both in the GI tract and at distal sites.

Both canonical and non-canonical autophagy pathways have been reported (82). We hypothesized that indole responses to lipotoxic stimuli are dependent upon canonical autophagy. Enhanced autophagy after rapamycin treatment (an mTor inhibitor and autophagy inducer) enables efficient clearance of lipids and prevention of foam cell formation.

Results

Role of atg5/autophagy in foam cell formation and atherosclerosis

During the autophagy, intracellular molecules and damaged organelles in the cytoplasm, degrade through a lysosomal route. Autophagy is a catabolic process that uses the contents of autolysosome for downstream metabolic pathways (75).

As mentioned in chapter 1, Macs uptake the ox-LDL and activate inflammatory processes; This leads to the production of inflammatory cytokines in the plaque area and recruiting other monocytes and Macs to the atherosclerotic lesion area (88).

Published data has indicated that autophagy inhibition, especially ATG5 autophagy, using chloroquine or using ATG-deficient Macs, leads to increased ROS production, inflammasome activation, and IL1-ß production, reverse cholesterol transport, accumulation of damaged organelles and proteins and cell apoptosis (Figure 56) (258).

Published data suggest the therapeutic intervention of autophagy inducers (especially in Macs) to prevent atherosclerosis (5,6). For example, Induction of LC3, ATG5, or Beclin-1 with pharmacological drugs can be good targets for the induction of autophagy.

In conclusion, activation of autophagy can be an excellent inhibitory candidate for inhibiting the pathogenesis of atherosclerosis (5,6).

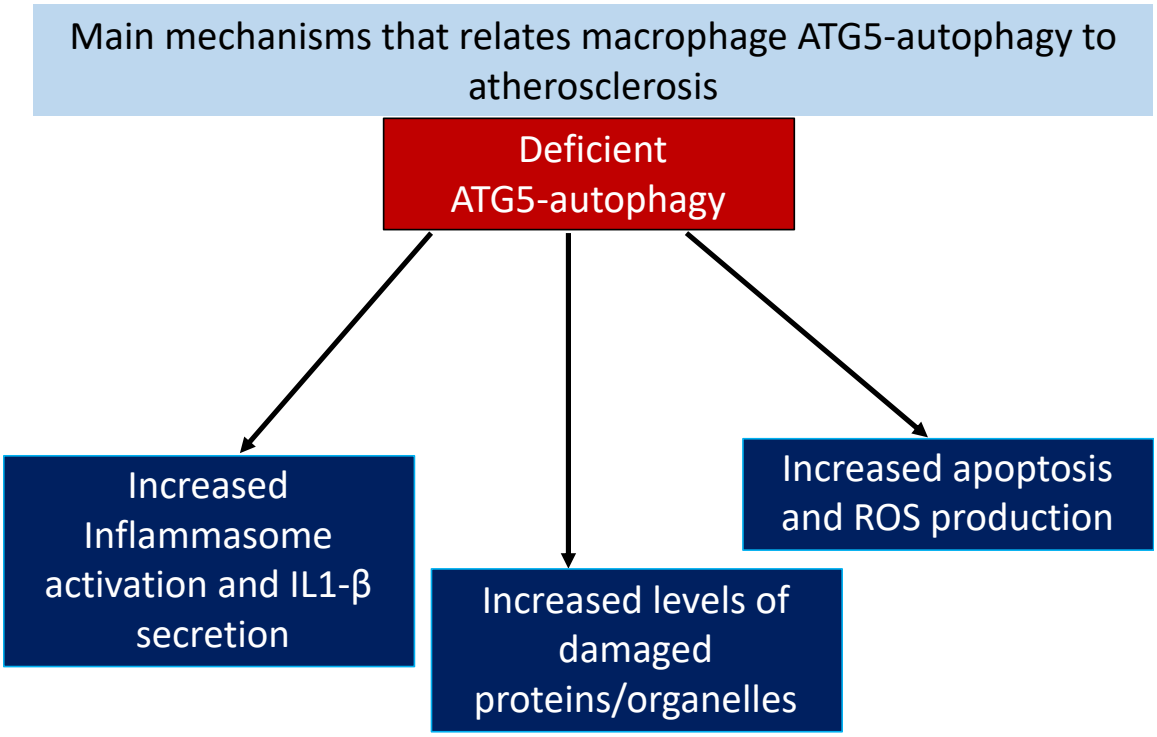


Figure 56. Role of atg5/autophagy in foam cell formation and atherosclerosis.

Inhibited atg5 autophagy increases atherosclerosis risk by increasing inflammation, increasing damaged organelles and proteins, and increasing apoptosis and ROS production.

Indole slightly increases LC3 gene expression in BMDMs.

LC3 or microtubule-associated protein 1A/1B-light chain 3 (LC3) is a protein that contributes to autophagosome membrane formation during autophagy (76). LC31 is a cytoplasmic form of LC3 that conjugates to phosphatidylethanolamine and forms LC3-2 that is recruited to autophagosome membrane. The LC3-2 to LC3-1 ratio is an indicator of active autophagy in the cells (76,257). Our previous lab data from western-blot indicated that indole pretreatment significantly increases the LC3-2/LC3-1 ratio. Confocal microscopy data indicated that indole increases the general amount of LC3 (data not shown), similar to rapamycin pretreatment.

We also tested the gene expression of LC3 in polarized BMDMs. We used day 7 BMDMs from WT or AhR-KO mice and pretreated them with indole or DMF solvent control overnight. Then we stimulated the cells with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of LC3, measured in polarized BMDMs using qRT-PCR.

Our results indicated that indole slightly (but statistically significant) increases the LC3 gene expression (Figure 57). This result is in line with our previous lab data regarding the indole's role in autophagy activation. Our data regarding the indole-induced inhibition of foam cell formation similar to the rapamycin-induced reduction of foam cell formation is another evidence of the indole-induction of autophagy in Macs.

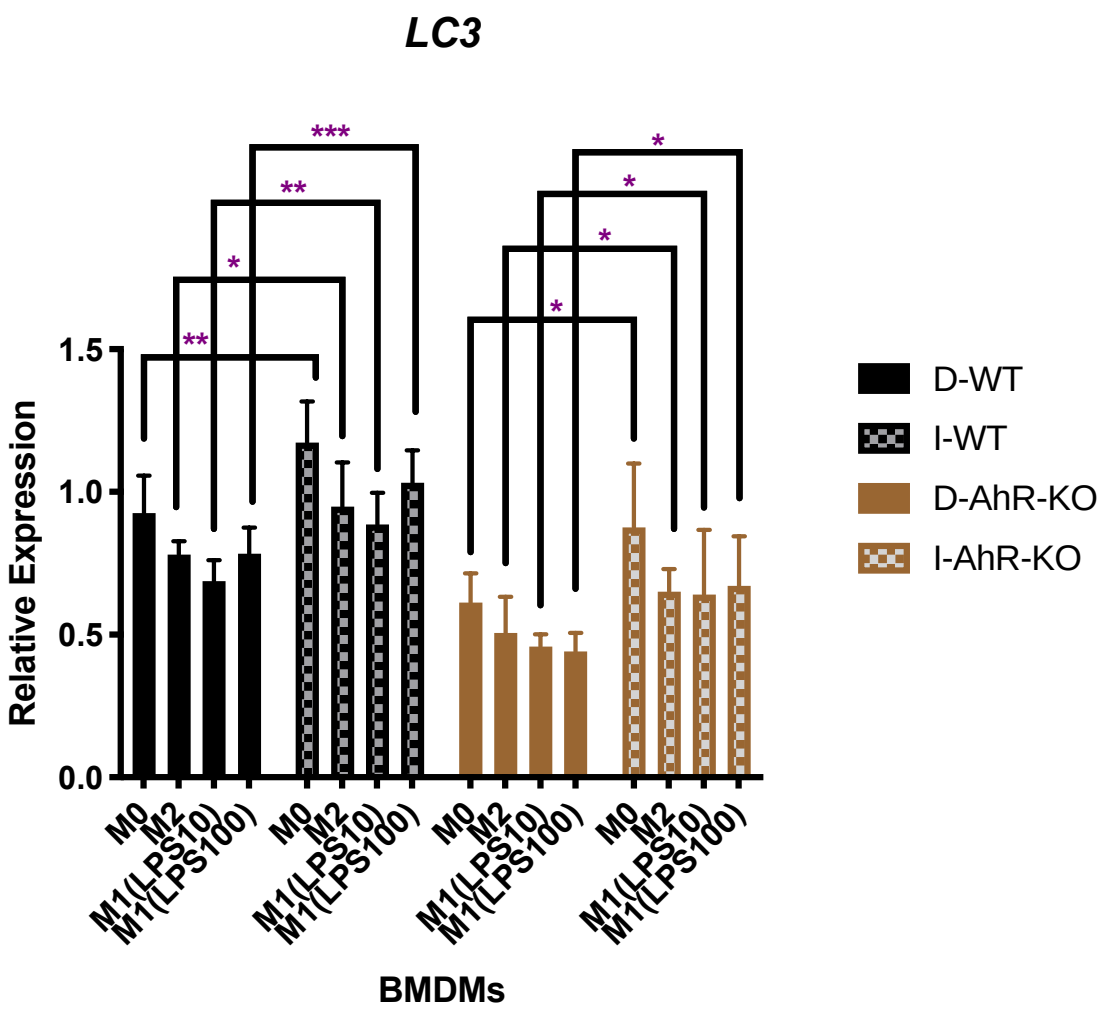


Figure 57. Indole slightly increases LC3 gene expression in BMDMs.

Day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of LC3, measured in polarized BMDMs using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Indole slightly increases Beclin-1 gene expression in BMDMs.

Beclin-1 is the mammalian ortholog of yeast ATG-6 and is involved in regulating autophagy and apoptosis. Beclin-1 mediates multiple vesicle trafficking pathways, especially autophagy (259). Beclin-1 binds to PI3KC3 (phosphatidylinositol-3 kinase) and forms a PI3KC3 lipid kinase complex that plays a role in autophagosome nucleation (260).

To test the gene expression of Beclin-1, we used day 7 BMDMs from WT or AhR-KO mice. Cells were pretreated with indole or DMF solvent control overnight and then stimulated them with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of Beclin-1 measured in polarized BMDMs using qRT-PCR.

Our result indicated that indole slightly (but statistically significant) increased the expression of Beclin-1 in all types of our polarized BMDM. The effects of indole were independent of AhR (Figure 58).

Our results were in line with our previous data, suggesting that indole activates autophagy.

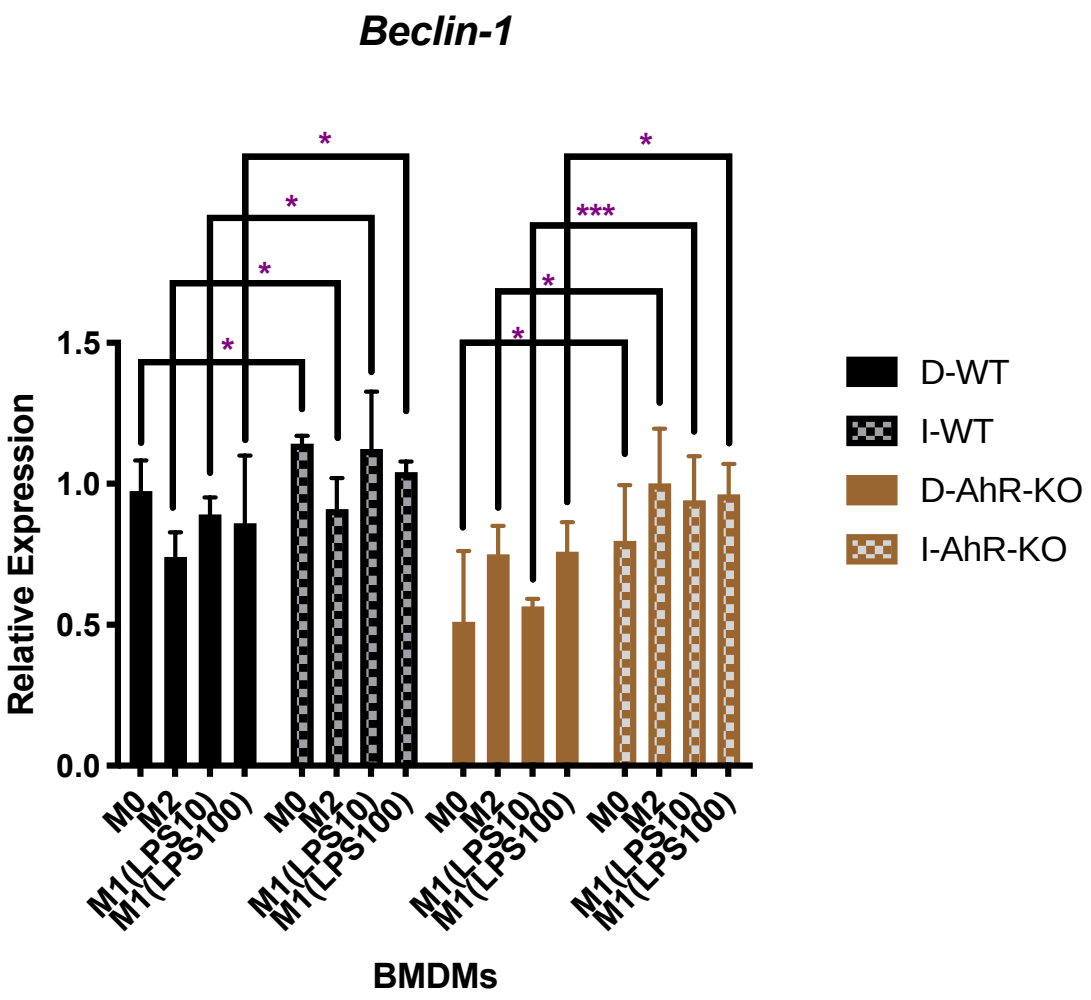


Figure 58. Indole slightly increases Beclin-1 gene expression in BMDMs.

Day 7 BMDMs from WT or AhR-KO mice were pretreated with indole or DMF control overnight then stimulated with polarization skews for 24 hours. M0 (Media), M1 (10ng/ml LPS) or M1 (100ng/ml LPS) and M2 (20ng/ml IL-4). Relative gene expression of Beclin-1 measured in polarized BMDMs using qRT-PCR. Data are representative of one of three experiments. "*": statistical significance between indole-treated and solvent controls using the Student's t-test at $p < 0.05$, "**", significance at $p < 0.01$, "***", significance at $p < 0.001$.

Role of atg5/autophagy in senescence and atherosclerosis.

Senescence is referred to as the cell cycle arrest and reduction of the replicative potential of the cells (261).

Senescence gets activated in different cells of atherosclerosis, in the early stages of the disease (261). Endothelial cells, vascular smooth muscle cells, and, importantly, Macs and monocytes are affected by senescence in atherosclerosis (261). Also, data has shown that foamy Macs accumulate senescence markers in the area of the atherosclerotic lesion (262,263).

Atherosclerosis and senescence both activate intermediate pathways that lead to inhibition of ATG-5 related autophagy (263).

On the other hand, activation of autophagy inhibits the progression of atherosclerosis and senescence (Figure 59) (262, 263). A mechanism or a pharmacological reagent that can inhibit senescence and activate autophagy can help autophagy regulation.

In the next section, we have tested the role of indole pretreatment in regulating hydrogen peroxide-induced senescence in Macs.

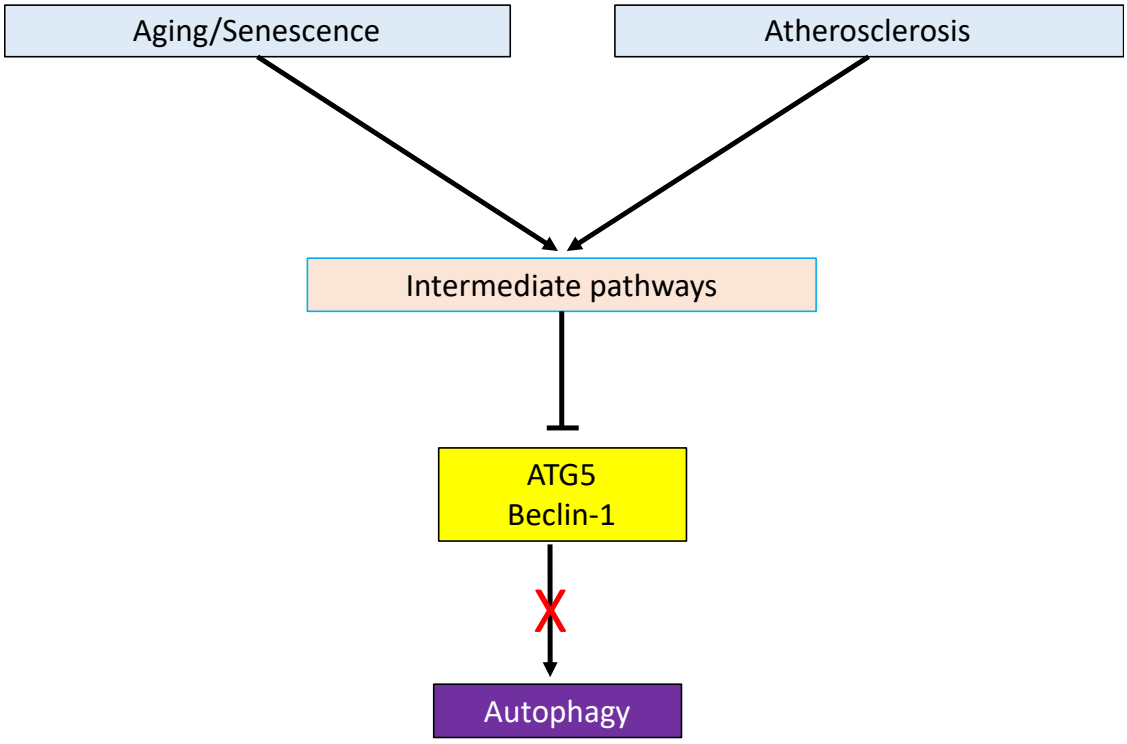


Figure 59. Role of atg5/autophagy in senescence and atherosclerosis.

Both senescence and atherosclerosis can inhibit atg5/Beclin-1 dependent autophagy through some intermediate pathways. Activation of the mechanisms that can induce autophagy in the cell (Macs) may reduce senescence and atherosclerosis progression.

Indole and rapamycin inhibit the senescence, and TMAO and I3S induce senescence in Raw264.7 Macs

Senescence limits the capability of the cell to replicate. Senescence is a state that cells remain viable with inhibited replication capacity (261-263). We cannot passage or divide the senescence cells in the culture. Senescence cells are larger and have an active (PH-dependent) beta-galactosidase activity. Also, senescence cells have changed the pattern of gene expression (264).

To understand the role of indole in senescence regulation, we performed β-galactosidase activity at pH 6 to detect senescent cells. We tested the role of TMAO and Indoxyl Sulfate as detrimental gut microbiota dependent and derived metabolites. We also used rapamycin as an autophagy inducer and positive control for indole.

We used different concentrations of hydrogen peroxide (H_2O_2) for 48 hours to induce senescence in Raw264.7 cell lines.

Cell lines were pretreated with indole or DMF control overnight (Day 0) then treated with TMAO, I3S, rapamycin or indole (on day1), and different concentrations of H_2O_2. On day2, cells were stained by β-Galactosidase Senescence Cell Staining, then analyzed by light microscopy. The development of the blue color is an indicator of senescence induction. Our results indicated that a low concentration of H_2O_2 was able to induce senescence, and indole pretreatment significantly prevented senescence induction. On the other hand, TMAO and Indoxyl sulfate (I3S) induced senescence even without the induction of H_2O_2. Rapamycin, similar to indole, inhibited the induction of senescence (Figure 60). Our results suggest that indole and rapamycin can be good candidates for inhibition of senescence. Probably, indole and rapamycin inhibit the senescence through the activation of autophagy.

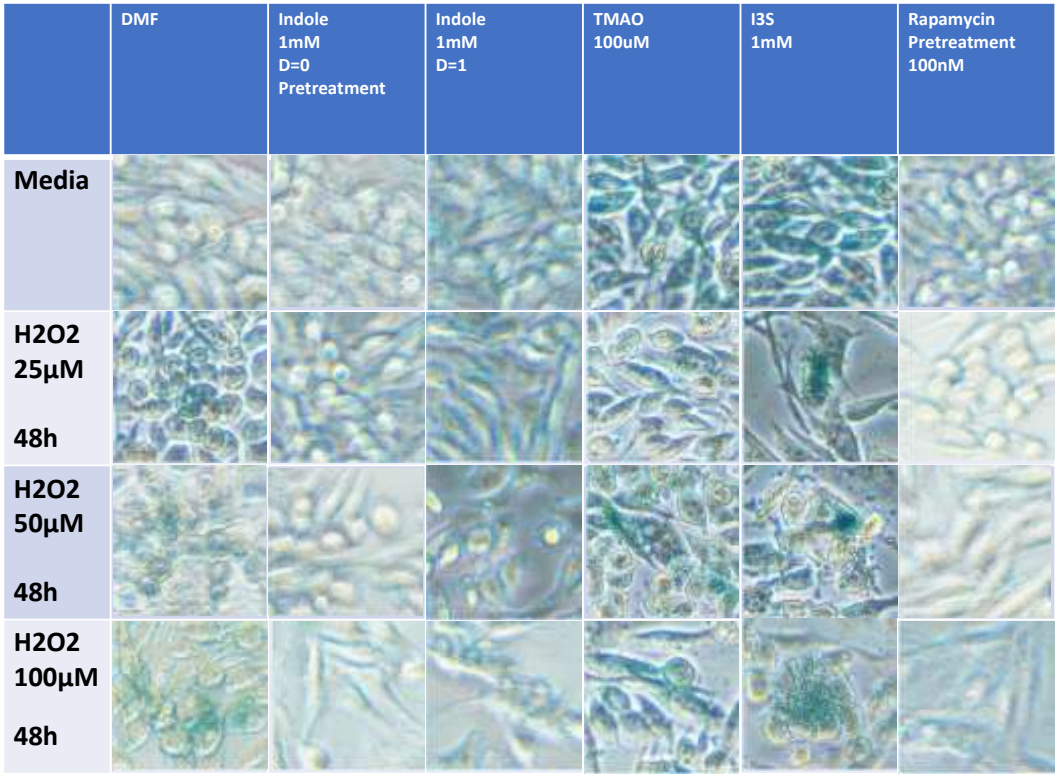


Figure 60. Indole and rapamycin inhibit the senescence, and TMAO and I3S induce senescence in Raw264.7 Macs.

Raw264.7 cell lines were pretreated with indole or DMF control overnight (Day 0) then treated with TMAO, I3S, rapamycin or indole (on day1), and different concentrations of H_2O_2 for induction of senescence. On day2, cells were stained by β-Galactosidase Senescence Cell Staining, then analyzed by light microscopy.

Our data concerning autophagy has indicated that indole can induce autophagy, similar to rapamycin. And this can control and reduce lipid accumulation, foam cell formation, and senescence activation. Table 3 is a color-coded list of the genes, proteins, and cell functions in the Macs. We have tested (AMPK protein information is from previous lab data) the role of indole in the autophagy-related factors. We have also mentioned the indole's regulation dependency on AhR.

Table 3. Table of indole's effects on Macs gene, protein, function in autophagy related factors.

Color coded role of indole:
Increase: M0, M1, M2
Decrease: M0, M1,M2
No Change: M0, M1,M2

Immuno-metabolism	Gene	Protein	Function	Dependency on AhR
LC3	**M0, M1, M2**			Independent
Beclin-1	**M0, M1, M2**			Independent
SIRT-1	**M0, M1, M2**			Independent
Lipophagy (compared to Rapamycin – Foam cell formation)			**M0, M1**	
Senescence (compared to Rapamycin – β-Galactosidase testing			**M0, M1**	
ATGL	**M0, M1, M2**			Independent
AMPK	M0, M2	**M0**		Independent

Indole regulates autophagy pathways related to foam cell formation.

The information in Figure 61. is similar to Table 3 but with the color-coded role of indole in autophagy pathways that are important in the process of Mac-derived foam cell formation.

In the previous chapters, we indicated that indole has anti-inflammatory roles in the regulation of Macs. We also showed that indole significantly inhibits the foam cell formation on BMDMs. And we tested some lipid metabolism pathways and the role of indole in regulating those pathways. Our data, in line with published data, suggest that active autophagy is essential in preventing atherosclerosis and foam cell formation (90).

The steps of autophagy have been demonstrated in Figure 61. top. My data and our previous lab data suggested that indole increases the AMPK gene expression and AMPK activation. AMPK activation inhibits downstream mTOR. Our unpublished lab data has also indicated that indole inhibits the mTOR, which can lead to activation of canonical autophagy (75-77). My data showed that indole increases the gene expression of Beclin-1 and LC3. Both of these genes are involved in the activation of autophagy (76, 259). Indole attenuates senescence, and this indirectly prevents the inhibition of autophagy in the cell.

Published data has indicated that lipophagy (autophagy of lipids) can significantly inhibit the foam cell formation (153, 265). Our data in chapter 3 suggested that indole and rapamycin inhibit foam cell formation, which is probably due to autophagy activation. Also, TMAO and 3MA (which is an inhibitor of autophagy) exacerbated lipid droplets' accumulation. In published data, activation of ATGL and SIRT1 has been suggested to have a role in the activation and induction of lipophagy (266). Our data in chapter 3 indicated that indole increases the expression of ATGL and SIRT-1 in all types of our polarized BMDMs.

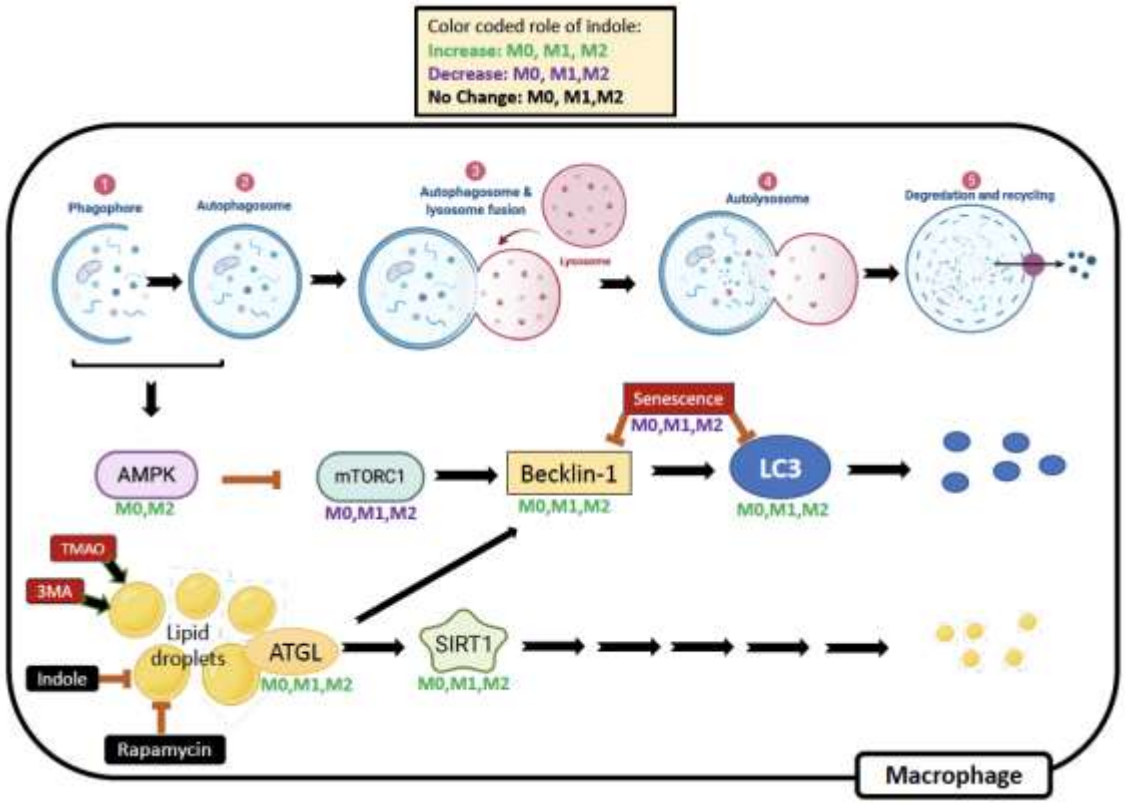


Figure 61. Indole regulates autophagy pathways related to foam cell formation.

The autophagy pathway is essential in preventing the Mac-derived foam cell formation process —the color-coded role of indole in the regulation of each metabolic element. ATGL, adipose triglyceride lipase; SIRT1, Sitrulin 1, an enzyme that deacetylates proteins that contribute to cellular regulation; AMPK, 5' AMP-activated protein kinase; LC3, Microtubule-associated protein 1A/1B-light chain 3 (LC3); mTOR, The mammalian target of rapamycin; Beclin-1, the mammalian ortholog of the yeast autophagy-related gene6 and BEC-1; TMAO, Trimethylamine N-oxide; TMA, Trimethylamine.

Discussion

Autophagy is a critical pathway in atherosclerosis (6). An important biomarker of canonical autophagy is converting microtubule-associated protein1 light chain 3 (LC3-I) to a lipidated form LC3-II, which localizes to autophagic membranes (267).

Notably, the amount of LC3-II within a cell reflects the amount of newly formed autophagic membrane and/or canonical autophagosomes (257). Our lab data show that indole induces autophagy similar to the canonical autophagy inducer rapamycin.

Since AMPK and mTOR regulate metabolism, which are important in atherosclerosis (81), we tested the indole regulation of AMPK in DCs and Macs. We found that indole activates and increases the gene expression of AMPK, which may link AMPK to indoles regulation of metabolism and autophagy.

Our data regarding the foam cell formation indicated that indole and rapamycin significantly inhibit the foam cell formation. 3MA, which is an inhibitor of autophagy, exacerbates the foam cell formation. These data first reflect the important role of autophagy activation in inhibiting lipid droplet accumulation and foam cell formation (90). Secondly, they suggest the role of indole in autophagy activation and, consequently, limit foam cell formation.

Our data indicated that indole slightly increases the gene expression of Beclin-1 and LC3, which are involved in autophagosome formation and autophagy activation (76, 259). Although the increase in gene expression was minimal, our previous lab data indicated a significant increase in protein levels of LC3II to LC3I ratio, similar to rapamycin, which is a strong indicator of autophagy activation induced by indole.

AMPK activation, LC3 expression, increased LC3II/LC3I ratio, and similar indole effects to rapamycin are all indicators of the indole induction of autophagy.

Autophagy of lipids (lipophagy) in the Macs is essential in the prevention of atherosclerotic foam cell formation. Published data suggest that increased levels of ATGL and SIRT1, along with the activation of canonical autophagy, can be an indicator of activation of lipophagy (266, 267).

Another factor that indirectly exacerbates the progression of atherosclerosis is senescence. During the senescence, cells go through some genetic alterations and lose their replication capability (262-264). On the other hand, through some intermediate pathways, senescence inhibits autophagy (263), which leads to increased levels of inflammation and foam cell formation in atherosclerotic plaques (263, 264).

Interestingly, our data indicated that indole and rapamycin inhibit the induction of senescence in the cells. TMAO and Indoxyl sulfate (I3S) that are addressed as detrimental gut microbiota dependent and derived metabolites, induced senescence in the cells even without the presence of hydrogen peroxide (inducer of senescence).

Overall, these data suggest the importance of autophagy in the down-regulation of foam cell formation and the prevention of atherosclerosis (6).

Published data indicate that ATG5 autophagy is not the only autophagy pathway in the cells (82). We highly recommend experiments in ATG5-KO cells to check the dependency of indole induced autophagy to canonical autophagy.

CHAPTER 5

CONCLUSION

Indole regulates Mac inflammatory responses to atherogenic stimuli

High-fat diet, saturated fatty acids, LPS induced inflammation, harmful microbiota metabolites such as TMAO, and ox-LDL are all pro-atherogenic stimuli (268).

Published data suggest that increased foam cell formation, reduced autophagy, increased inflammation, reduced fatty acid oxidation, and increased aerobic glycolysis in Macs can make them prone to turn into foam cells, which can accumulate in atherosclerotic lesions and form plaques (97).

Macs, similar to dendritic cells, are the cellular junction bridging innate and adaptive immunity (269). Dendritic cells are a crucial immuno-regulatory cell in the gastrointestinal tract (270), and Macs are the most important immune cells associated with atherosclerosis (271).
Our study provides in vitro evidence that the microbiota-derived metabolite, indole, plays a role in down-regulating the High-fat-diet induced, saturated fatty acid-induced (SFA) and LPS-induced inflammatory changes in Macs and dendritic cells.

Indole regulated the polarization of Macs (M0, M1 & M2) in response to pro-atherogenic stimuli

There are a variety of Mac phenotypes in atherosclerotic plaques (116). When monocytes in the circulation encounter modified LDLs and atherosclerotic lesions, they penetrate the plaque area (115-117). Then they turn into naïve Macs. Based on the signals that these Macs get from their environment (115); they can polarize to different Mac phenotypes with other metabolic and physiologic characteristics (116).

Most atherosclerotic Macs are M1 or pro-inflammatory ones. LPS, ox-LDL, and inflammatory cytokines are examples of M1 Macs stimulators (19).

M1 Macs have an active fatty acid synthesis metabolic system, and they indicate reduced levels of lipid catabolism and are the most prone Macs to transform into foam cells (97). Also, M1 Macs recruit other Macs to the plaque area (193).

On the other hand, M2 Macs indicate anti-inflammatory characteristics and have a role in the plaques' stability (19).

Our data regarding the indole's role in Macs ' polarization indicated that indole reduces most of the M1(CD38, MCP-1, glycolysis) polarization markers. Indole increases iNOS, which is an M1 marker, but we hypothesize that indole induced iNOS expression is because of the indole's bacterial killing properties. On the other hand, indole reduces (CD206, Egr2, Arg-1) and increases some M2 (PPAR-γ, AMPK, STAT3)- markers. These data suggest that indole is not completely switching the M1 phenotype to the M2 phenotype. It is actually inducing a specific phenotype that has bacterial killing properties and reduced inflammatory states. In chapters 3 and 4, we have indicated that indole pretreatment increases lipid catabolism and reduces the lipid anabolism in the Macs. Also, indole induced Mac phenotype indicates active autophagy.

Our data indicated that detrimental gut microbiota dependent metabolite, TMAO, and gut microbiota-derived metabolite Indoxyl sulfate, significantly increase M1 polarization of Macs and indole inhibits TMAO or Indoxyl sulfate-induced M1 polarization.

Indole regulates the metabolism of Mac responses to atherogenic stimuli

Atherogenic stimuli such as LPS and saturated fatty acids induce M1 polarization of Macs (97). Increased amounts of glycolysis and reduced amounts of the OXPHOS pathway indicate M1 Macs metabolic states (97). Indole pretreatment reduced the glycolytic characteristics of M1 Macs,

but on the other hand, indole reduced OXPHOS levels. That is why we became interested in checking fatty acid oxidation as an ATP production pathway (97, 138). Our data indicated that indole activates fatty acid oxidation pathways. This helps the cell catabolize the stored lipids in the cells and provide energy through the beta-oxidation pathways. Our other data regarding the effects of indole on Macs ' lipid metabolism was in line with our bioenergetic data. Indole activated lipid catabolism pathways (increased, lipolysis, FAO, and cholesterol efflux). It reduced lipid anabolic pathways (reduced FA synthesis, lipid droplets accumulation, and foam cell formation). With this specific metabolic phenotype, indole can regulate foam cell formation of Macs; This can have a huge effect in controlling and preventing atherosclerosis.

Indole regulates Mac metabolic phenotypes and inflammatory responses independent of Aryl hydrocarbon receptor (AhR)

Although our result indicates novel metabolism and indole mechanisms in Macs and APCs, the initial mechanism of action of indole in Macs and APCs is still unknown. Although indole is one of the ligands for AhR (48), our data indicated that AhR-KO Macs have suggested that the effects of indole in Macs (and previously showed that in dendritic cells), is entirely independent of AhR. Since indole is a small molecule and easily diffusible, its mechanism of action can be through other unknown surface or intracellular receptor (s) (50). To this end, we highly suggest future studies regarding the receptors and mechanisms of activity of indole.

Based on our lab data (data not shown), It is important to mention that the anti-inflammatory effects of indole are AhR dependent in T-cells. Data suggests that AhR expression levels and AhR's affinity to indole or other ligands may be different, depending on cell type (272). Transcriptomic studies can be useful in comprehensive studies of indole related pathways cascade.

Indole inhibits oxLDL induced foam cell formation in Macs

Accumulation of lipid-laden Macs or foam cells in atherosclerotic lesions is one of the main reasons for plaque formation (237, 238).

Therapeutics that can target and inhibit foam cell formation can be very useful in preventing and treating atherosclerosis (116). Indole is an endogenous gut microbiota-derived metabolite that can significantly impede the foam cell formation in vitro. Indole can be an excellent therapeutic candidate, with minimal side effects for the body. On the other hand, understanding indole's mechanism of action, in inhibition of foam cells, can help design drugs that can target those specific pathways. For example, activation of autophagy and lipophagy, cholesterol efflux, fatty acid oxidation, and lipolysis; and inhibition of inflammation, fatty acid synthesis, and Macs ' M1 polarization, can significantly reduce the lipid droplets in the Macs (153, 155).

Indole regulation of lipid metabolism in the Macs is independent of AhR

Some published data and our previous lab data in T-cells indicated that the mechanism of action of indole is through the Aryl hydrocarbon receptor (273). But our data regarding the effect of indole on Macs and dendritic cells metabolism in WT and AhR-KO cells is completely AhR independent. We have examined immunometabolism and lipid metabolism in Macs, and we found out that indole has a significant role in the regulation of lipid metabolism. But our experiments in AhR-KO Macs indicated that indole regulation of lipid metabolism is entirely AhR independent, which suggests that indole is probably acting through other surface or intracellular receptors.

Indole induces autophagy in Macs

Indole mediates cell metabolism through different pathways. One of the important pathways that regulate immune metabolism and lipid metabolism is autophagy (6). Rapamycin is a potent autophagy activator in the cells (152). The role of active autophagy in the inhibition or

prevention of atherosclerosis is documented (256). Since in canonical autophagy, mTOR is a target of rapamycin (82), and since the effects of indole on Macs were very similar to rapamycin, we predict that indole induces anti-inflammatory, bacterial killing, and lipolysis effects through mTOR/autophagy activation. Also, when autophagy is active in the cell, damaged intracellular organelles, pathogens, and proteins will be degraded, and this process will provide extra energy and increased survival for the cell (274). Our lab data (data not shown) has shown that indole, similar to rapamycin, inhibits phosphor-S6, which is a downstream target of mTOR; this suggests that rapamycin and indole inhibit mTOR activity. On the other hand, our previous lab data indicated different indole and rapamycin effects on cytokine production of APCs. For example, rapamycin increased IL-12 production and inhibited IL-10, but indole inhibited IL-12 and did not alter IL-10 in similar conditions (data not shown). These data suggest that the mechanism of action of indole, as well as its metabolic effects, is not entirely identical to rapamycin, even though indole and rapamycin act similarly in the activation of autophagy. That is why a comprehensive study of the indole's activation of autophagy, and its comparison with rapamycin activation of autophagy, can unravel more accurate and detailed information about the similarity and differences of indole and rapamycin activated pathways. It is important to mention that indole is an endogenous gut microbiota-derived metabolite. In comparison, rapamycin is produced in a soil-dwelling bacterium (275).

Future directions

A better understanding of the specific molecular pathways is required to develop appropriate therapeutic agents. Studies indicate that intestinal microbiota can have significant effects on metabolic disorders such as atherosclerosis (174). Intestinal microflora exhibits both

anti-atherogenic and pro-atherogenic effects (276). Withal, this research is only one instance of the unique interaction between microbiome and health/disease.

We highly suggest future studies to implement atherosclerosis animal models (i.e., ApoE-/- and LDL-receptor null mice) to investigate the link between microbiota dependent metabolites and cardiovascular diseases (CVDs). Besides, our study aims to reveal the therapeutic potential of microbiota metabolites, to limit inflammation, inhibiting foam cell formation, and promoting autophagy to develop treatment options for CVDs.

Closing remarks

Macs are the critical cells in the pathogenesis of atherosclerotic. They have an important role in plaque formation in atherosclerotic lesions. During atherosclerosis, monocytes in the circulation penetrate the arteries' atherosclerotic lesions and turn into naïve Macs. The naïve Mac, based on their microenvironment signals, get polarized towards different types of Mac phenotypes.

The most popular phenotype of Macs in atherosclerotic plaques is the M1 phenotype. These Macs are inflammatory and prone to transform into lipid-laden foam cells. Inhibited autophagy, reduced lipid metabolism, and Macs' active inflammation have an important role in foam cell formation.

So far, very few microbiota metabolites have been identified that have significant roles in regulating atherosclerosis pathogenesis and progression in the host. Gut microbiota dependent TMAO is known to have detrimental effects on atherosclerotic patients. On the other hand, Indole is a gut microbiota-derived metabolite reduced in plasma levels of atherosclerotic patients. In this research, we demonstrated that Indole induced signals could modulate Macs characteristics towards a phenotype that has active autophagy levels and reduced inflammation and increased lipid catabolism (Figure 62).

Studies in this research unravel data regarding the link between microbiota and atherosclerosis, which has not been thoroughly investigated before. Indole induces an anti-atherogenic phenotype in Macs, which can be incorporated as a future candidate for endogenous therapeutics to prevent and treat atherosclerosis.

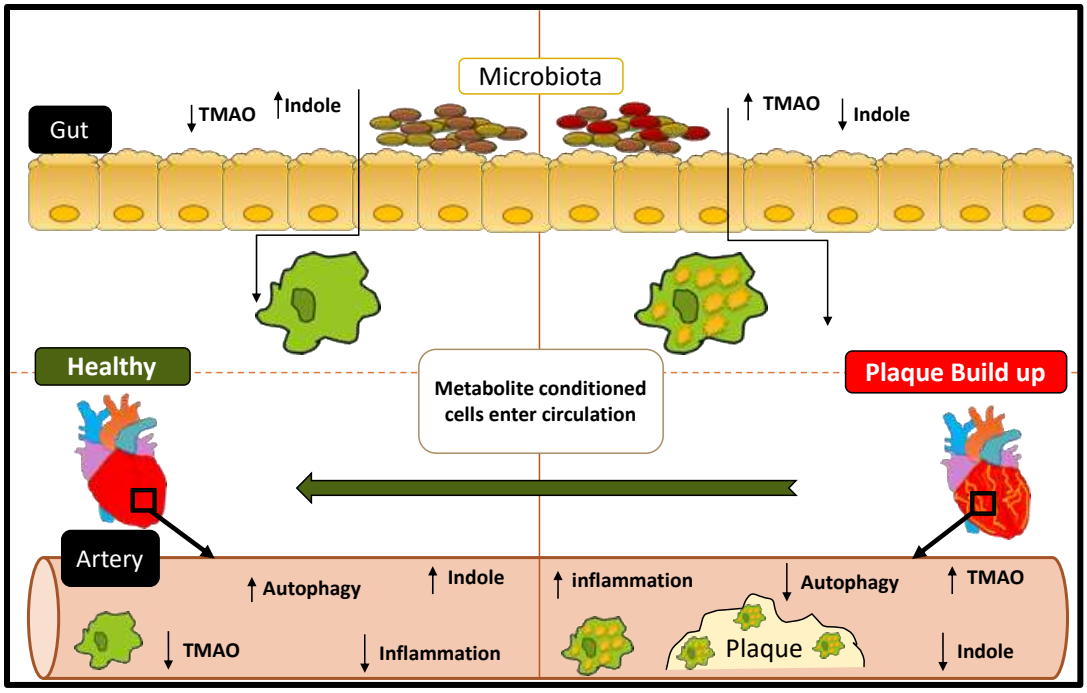


Figure 62. Indole regulates autophagy, inflammation and lipid metabolism pathways.

We have established a new modeling strategy to understand part of the unknown dynamics between microbiota metabolites, and Mac metabolism in atherosclerosis disease model.

CHAPTER 6

METHOD'S SUMMARY

Methods

Mice

We used C57BL/6 (WT) mice, purchased from Jackson Laboratory (Bar Harbor, ME). Also, we used AhR-KO mice that were purchased from Taconic Farms (Rockville, MD). Mice were located and maintained in a specific pathogen-free animal facility located at Texas A&M University Health Science Center (Reynolds Medical Building). All mice that we used were age and gender-matched for each experiment, and the ages were between 6 and 12 weeks.

Mice were humanely sacrificed according to Assessment and Accreditation of Laboratory Animal Care (AAALAC) and American Association for Laboratory Animal Science (AALAS) guidelines and using protocols approved by the Texas A&M University institutional animal care and use committee (IACUC).

Reagents

We purchased indole from Sigma (St. Louis, MO). Dimethylformamide (DMF) was used to dissolve indole and added to cultures at indicated concentrations. We purchased Recombinant murine GM-CSF and MCSF from Preprotech (USA). Ultra-pure LPS was ordered from Invitrogen (San Diego, CA) and was used for Mac and DC activation. Antibodies used for flow cytometry are indicated in Table 4.

Table 4. List of the antibodies used in this study.

Antibody	Clone	Company	Cat#
CD11b	M1/70	Invitrogen	48-0112-82
F4/80	BM8	Invitrogen	25-4801-82
CD11c	N418	eBioscience	12-0114-83
TNF	MP6-XT22	BD	558000
CD86	GL1	BD	560581
IL-12	p40/p70	BD	554480
CD38	90/CD38	BD	553764
CD206	MMR	VWR	141710-BL
iNOS	CXNFT	Invitrogen	53-5920-82
Arg-1	AlexF5	Invitrogen	25-369782
CD36	CRF D-2712	BD	7198756
Egr-2	erongr2	Invitrogen	17-6691-80

Primary cells culture

Mac and DC primary cells were cultured in RPMI 1640, supplemented with 2-mercaptoethanol, gentamicin, penicillin, streptomycin, and 10% FCS (all from Life Technologies). Bone marrow-derived dendritic were harvested from murine femurs and cultured in RPMI-complete media supplemented with 10% FCS, 20ng/ml GM-CSF (Peprotech). Media was replenished on day 3 and 5 of the culture, which was seven days in total. Indole or DMF solvent control was added to the cultures before the LPS stimulation or polarization.

In Mac culture, bone marrow was harvested as described above and cells cultured at a density of 2×10^6 in 10ml complete media supplemented with 10ng/ml M-CSF (Peprotech) in 100mm x 15mm Petri dishes. Media with cytokine was replenished on day 3. Adherent and semi-adherent cells were collected with cold PBS (without calcium and magnesium) on day 7 (Figure 63). Then the cells plated and treated with indole or solvent control prior to downstream experiments. Stimulation of cells at the given concentrations of LPS was performed for 4 hours with the presence of Golgi-Plug protein transport inhibitor (BD Biosciences) or 12 hours without Golgi-Plug in to measure polarization.

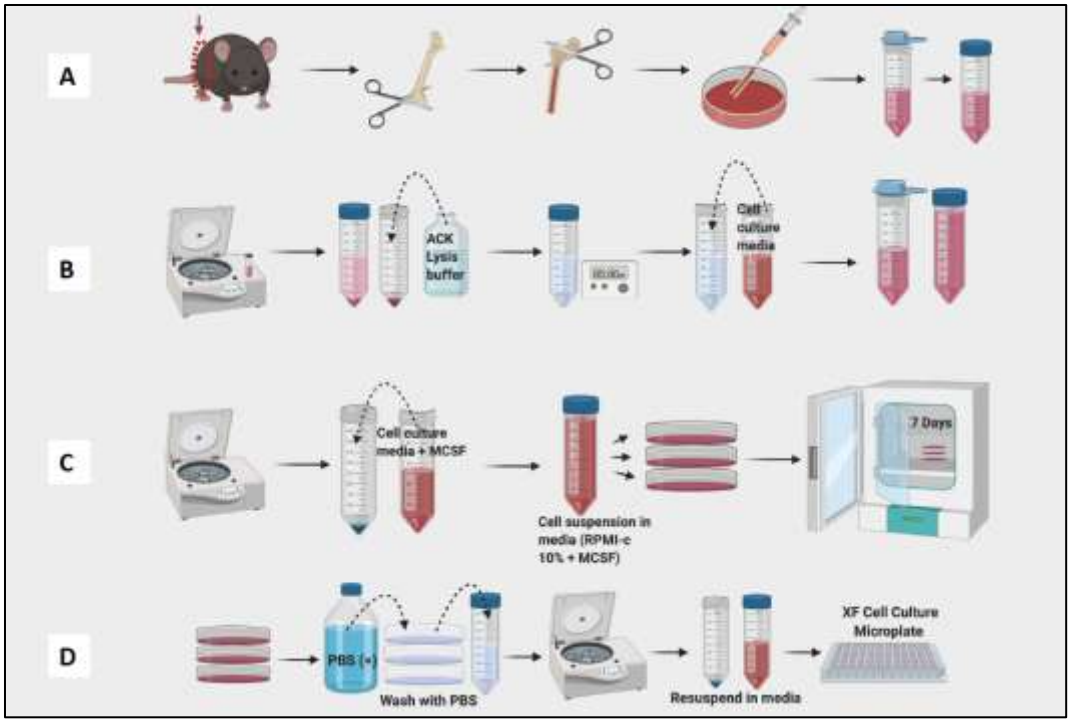


Figure 63. Graphical workflow of mouse bone marrow culture of BM-Derived Macs.

(A) Leg harvest, Femur exposure and marrow flush; (B) RBC Lysis; (C) Plating and culture; (D) Cell harvest from the plates.

Mac polarization

10ng/ml LPS, added (to the Mac cell media) to polarize the naïve Macs towards M1, and 20 ng/ml of IL-4 used to polarize the Macs towards M2. Then the cells plated in an incubator at 37ºC 5% CO_2 for 24 hours.

Raw264.7 Macs

Dr. Paul Defigueiredo kindly provided the murine Raw264.7 Macs, Texas A&M University, Health Science Center. Raw264.7 cells were cultured in complete RPMI medium supplemented with 5% fetal calf serum (FCS).

Single cell suspension of Spleen cells

Following dissection, spleens submerged in their designated 5 ml medium containing tubes, RPMI-1640 medium supplemented with 2-mercaptoethanol, gentamicin, penicillin, streptomycin, and 10% FCS (all from Life Technologies) and arranged on ice.

Spleens homogenized between 2 frosted microscope slides. Samples were incubated in 5ml ammonium-chloride-potassium (ACK) buffer for 5 mins (Cambrex, Walkersville, MD) to remove blood cells and passed through a 70 mm cell strainer (Falcon) simultaneously with 10 ml of medium. After centrifugation, cell pellets reconstituted in RPMI-1640 media. Cell numbers were determined using trypan blue (SIGMA-ALDRICH) and counted on a hemocytometer. Heterogeneous spleen cells were plated and incubated for 4 hours in the presence or absence of Golgi-Plug protein transport inhibitor (BD Biosciences) in 37^{o}C 5% CO2 incubator. Cultures were stained for multicolor flow cytometry analysis.

Flow Cytometry

Cells were washed in PBSA buffer (PBS + 5% BSA (SIGMA-ALDRICH)). Prior to staining, samples were incubated with FCR Blocking Reagent (BD Biosciences). Surface antibodies were diluted in FACS/PBSA buffer and incubated with samples for 30 min on ice, followed by washing with FACS buffer. After washing in PBSA buffer, cells were fixed in 4% paraformaldehyde (SIGMA-ALDRICH) in PBSA. Intracellular staining was performed to assess intracellular markers and cytokines using Permeabilization buffer (BD Biosciences). Cells re-suspended in PBSA buffer and stored at 4 °C until analyzed.

Flow cytometry performed using a Fortessa flow cytometer using DIVA version 8 software. The analysis performed using FlowJo software version 10.1r5.

Mito-trackers experiments

BMDMs were cultured in a 96 well plate as described above. Then they pretreated with indole/DMF/oligomycin for 24 hours.

MitoTracker Green was used for total mitochondrial mass identification, MitoTracker Red used for mitochondrial membrane potential detection, and MitoSOX used for mitochondrial ROS staining.

Experiments were performed based on the manufacturer's instructions (Invitrogen). Data were acquired with a FACS flow cytometer (BD Fortessa) and analyzed with FlowJo software for flow cytometry analysis.

Real-time PCR

According to the manufacturer's instructions, to assess the mRNA expression, total RNA was isolated from BMDMs using Aurum Total RNA Mini Kit (Bio-Rad). Quality and quantity of the RNA measure by nanodrop (Thermo Fisher Scientific).

In order to make cDNA, the retro transcription performed using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). 500ng/ml of RNA used to make complementary DNA. cDNA-PCR reaction carried out in a 96 well thermocycler (Eppendorf master cycler pro 6321). In order to eliminate genomic DNA contamination, RNA samples were treated with RNase-free DNase (Ambion, Austin, TX, USA).

Quantitative real-time PCR reactions were performed in triplicate using the SYBR Green PCR Master Mix. The reaction ran on a 384 well plate Bio-Rad CFX384 model (Bio-Rad Lab., Hercules, CA, USA).

The primer sets are explained in (Table 5). Results are the average ± standard error of at least three independent biological samples, and quantification of gene expression levels calculated using the ΔCt method. Gene expression levels were normalized to Cyclophilin-A as the housekeeping gene for each of the individual samples.

Table 5. List of the primers used in this study.

Gene	Forward	Reverse
Cyclophilin	CTAAAGCATACAGGTCCTGGCATCTTG	TGCCATCCAGCCATTCAGTCTTG
CD38	TTGCAAGGGTTCTTGGAAAC	CGCTGCCTCATCTACACTCA
MCP-1	TCAGCCAGATGCAGTTAACGC	TGATCCTCTTGTAGCTCTCCAGC
Chitinase 3-like 3	GGCTCAAGGACAACAATTTAGG	ACTGTGGAAAAACCGTTGAACT
Arginase 1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
Resistin like alpha	TATGAACAGATGGGCCTCCT	AGCTGGGTTCTCCACCTCTT
Atp5b	GGTTCATCCTGCCAGAGACTA	AATCCCTCATCGAACTGGACG
FAS	TGG GTT CTA GCC AGC AGA GT	ACCACCAGAGACCGTTAT GC
CPT1a	ACGCATGACAGCACTGGCCC	CCTCCCCAGGGATGCGGGAA
ATGL	CAGCACATTTATCCCGGTGTAC	AAATGCCGCCATCCACATAG
ABCG1	CTTTCATGTCCCGCTGCAT	GTCACGGGACCCACAAATG
iNOS	TCCTGGAGGAAGTGGGCCGAAG	CCTCCACGGGCCCGGTACTC
CD206	TGATTACGAGCAGTGGAAGC	GTTCACCGTAAGCCCAATTT
Egr2	AACGGAGTGGCCGGAGAT	ATGGGAGATCCAACGACCTCTT
PPAR-γ	TCTTAACTGCCGGATCCACAA	GCCCAAACCTGATGGCATT
NADH	TACACGATGAGGCAACCA	GGTAGGGGGTGTGTTGTG
SIRT-1	AACGTCACACGCCAGCTCTA	CCAATCATGAGATGTTGCTGAAC
FoxO	GCGTGCCCTACTTCAAGGATAA	TCCAGTTCCTTCATTCTGCACT
Nrf-2	ATGCCAGCCAGCTGACCT	AGACGGTGGCAGCATGCC
HO-1	GCCGAGAATGCTGACTTC	TGGTACAAGGAAGCCATC
AMPK	AAGCCGACCCAATGACATCA	CTTCCTTCGTACACGCAAAT
LC3	CCGTCCGAGAAGACCTTCAAGCAGC	TTGTTGGAGTCTTACACAGCCATTG
Beclin-1	GTTGCCGTTATACTGTTCTG	CCTCCAGTGTCTTCAATC
Glut-1	TGTGGTGTCGCTGTTTGTTGT	CCTCGGGTGTCTTGTCACTT

NAD quantification Colorimetric assay

We used NAD Quantification Colorimetric Kit (Bio Vision) to test the total intracellular nicotinamide adenine dinucleotide NAD+ based on the manufacturer's protocol.

Lactate Assay

In order to measure lactate, BMDMs polarized based on the information in the polarization section, and lactate levels were quantified using the Lactate Assay Kit from Sigma-Aldrich (MAK064), according to the manufacturer's recommended instructions.

β -Galactosidase assay

In order to measure senescence in Raw264.7 Macs, after pretreatment of indole/DMF, TMAO, and indoxyl sulfate (25, 50, and 100 μM), H_2O_2 (Sigma) added to the media for 48 hours to induce senescence in the cells.

β-Galactosidase levels were detected using the Senescence β-Galactosidase Staining Kit from Cell Signaling Technology (9860) and light microscopy, according to the manufacturer's recommended instructions.

The microscopic photography performed using Nikon eclipse TS 100 and processed with NIS Elements 4.0 software.

Seahorse Metabolic analyzer

80K or 50K Cells were cultured in an Agilent Seahorse XF_e96 microplate per well. The Seahorse XF_e was turned on and warmed up to 37^OC on the day before the assay.

A seahorse cartridge was hydrated by adding 200μl of Seahorse Calibrant Solution (Cat #103059-000) and located in a non-CO_2 incubator overnight on the day before the assay (Figure 64. A). Cultured cells settled in BSC hood for 45 minutes in RT to prevent the edge effect of the plate on

cells and pretreated with indole (1mM) or DMF as a control for 24 hr, then treated with/without polarization skews 24 hr. 3 to 6 replicates were plated per sample (Figure 64. B).

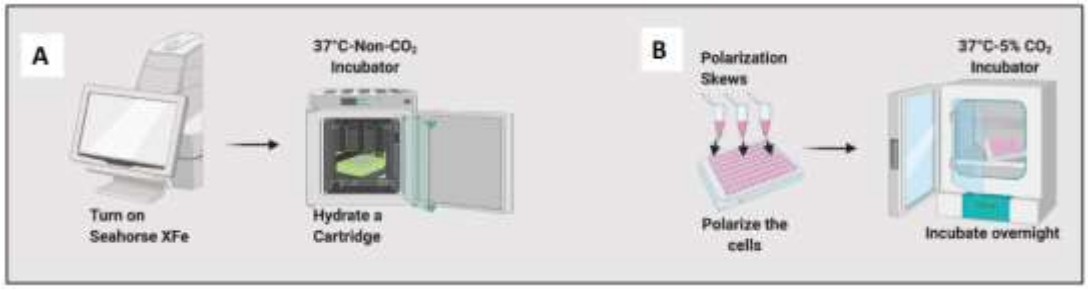


Figure 64. Graphical demonstration of seeding and polarization of the cells.

(A) Seahorse XF_e Set up cartridge hydration; (B) Polarization of the cells and overnight incubation.

Seahorse glycolytic assays

100ml of XF RPMI (pH 7.4) assay medium was completed with 2mM glutamine. The media filter-sterilized using a 0.2µm vacuum filter system. The assay media Placed in a 37^{o}C water bath for 20 min. The plated cells were removed from the 37oC 5% CO2 incubator, and the cells were washed with assay media twice, and the previous media replaced with Seahorse assay media to the final volume of 180µl. The cell-containing plate Located in a non-CO2 incubator for 45 min (Figure 65. A.).

Using the compounds provided in the XF Seahorse glycolysis stress test kit, Glycolytic rate assay kit, and the assay media, stock solutions of Glucose (100mM), Oligomycin (100µM), Rot/AA (50µM), and 2DG (500mM) were made (Table 6).

Make a 10X injection mixture of each compound using assay media (Table 7).

Table 6. List of the injection stocks.

Injection Stocks (Provided in the kits)	Add Complete assay media (ml)	Final Stock concentration(µM)
Glucose	3	100k
Oligomycin	0.72	100
Rotenone/ antimycin A	0.54	50
2-DG	3	100k

Table 7. List of the injection stocks.

Ports on the Cartridge	Stock solutions	Add stock volume		Add Assay media	Final concentration of injections (10X)	Add this volume to designated port	Final concentration after injection in each well
A	Glucose (100 mM)	3000µl	+	0µL	100 mM	20	10 mM
B	Oligomycin (100 µM)	300µl	+	2700µl	10 µM	22	1.0 µM
C	Rotenone / antimycin A (50 µM)	300µl	+	2700µl	5 µM	25	0.5 µM
D	2-DG (500 mM)	3000µl	+	0µL	500 mM	28	50 mM

On the assay day, the glycolysis test was performed on polarized BMDMS, using a modified acute glycolysis stress test template in wave software. The injection names were adjusted based on the table Table 6.4**.** and after clicking the run bottom, everything followed based on the default instructions. Then the cells plate removed from the non-CO_2 incubator and inserted in the Seahorse instrument (Figure 65. B).

By using the CyQUANT cell proliferation assay kit, the cells were normalized based on naïve Macs cell count (Figure 65. C**).** naïve Macs average considered as one, and their average number applied to all Macs.

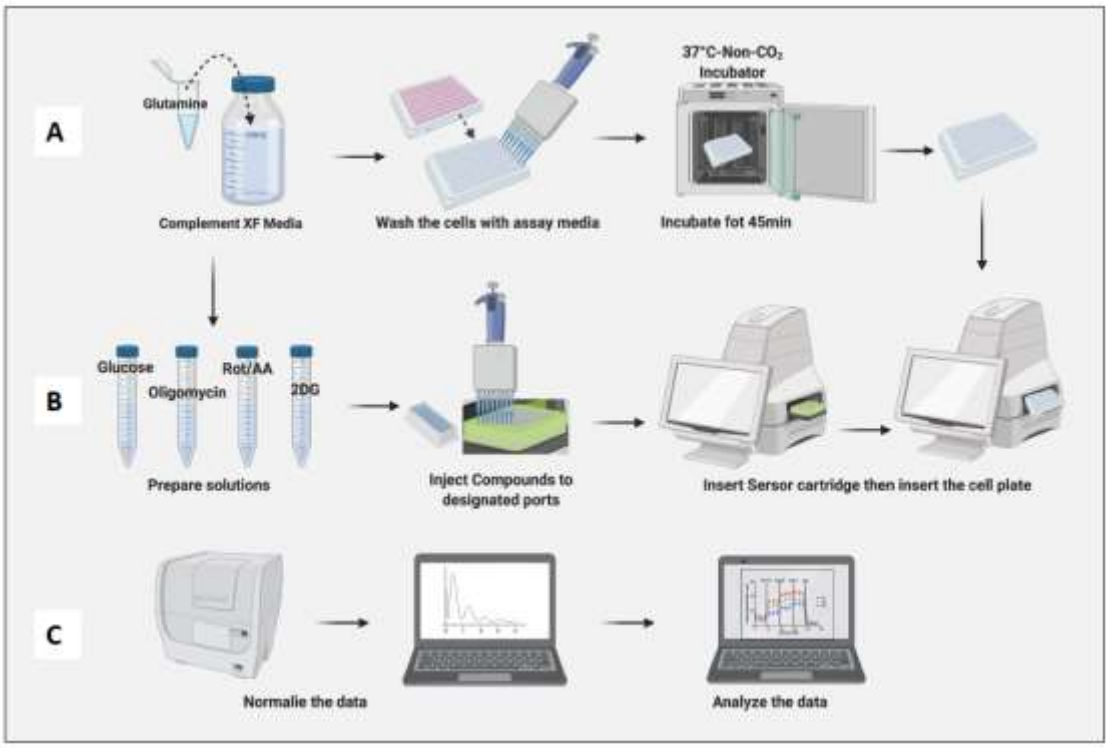


Figure 65. Day of the Assay: Medium and compound preparation and running the assay.

(A) Cells preparation for assay; (B) Compounds preparation, calibration, and running the assay; (C) Normalization and data analysis.

Glycolysis Stress test

To perform the glycolysis Stress Test, the cells were pretreated with Indole or solvent control and then polarized in the seahorse microplate. The rest of the experiment was performed based on the manufacturer's instruction. The Glycolysis Stress Test data generator, in wave software, is used to generate data.

Seahorse Mito-Stress Test

Seahorse Assay Media was supplemented with a final concentration of 10mM glucose, 1mM pyruvate, and 2mM glutamine (All from Agilent technologies). On the day of the assay, cell

culture media was changed to seahorse assay media, and the plate was located in a non-CO_2 37^oC incubator for an hour.

Using the seahorse Mito Stress Test kit (Agilent technologies), Oligomycin was added to port A for a final concentration of 1uM. FCCP was added to port B for a final concentration of 1µM. Rotenone/Antimycin (0.5µM) added to ports C. All injectables diluted in the seahorse assay media. Wave software was used for performing the assay and analyzing data. XF data generator and GraphPad Prism was used for making the graphs.

Basal respiration shows the energetic demand of the cell under baseline conditions (Seahorse Bioscience Glossary). The maximal oxygen consumption rate is attained by adding the uncoupler FCCP. FCCP mimics a physiological "energy demand" by stimulating the respiratory chain to operate at maximum capacity, which causes rapid oxidation of substrates (Sugars, fats, amino acids) to meet this metabolic challenge. Shows the maximum rate of respiration that the cell can achieve (seahorse bioscience glossary). The seahorse assay was performed in the college of medicine cell analysis center (COM-CAF).

Seahorse ATP Assay

Raw264.7 cells were cultured and polarized in a seahorse microplate with the details as mentioned above. Then the assay was performed based on the manufacturer's instruction.

Seahorse FAO Assay

Day 7 BMDMs harvested and plated in a seahorse microplate. The cells pretreated with 1mM indole or DMF solvent control. Then seahorse FAO assay was performed based on the manufacturer's protocol.

Table 8. List of Seahorse related material.

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Fetal Bovine Serum	Life Technologies	16000-044	
Kim Wipes	VWR	82003-822	
ACK lysis buffer	Thermo Fisher Scientific	A1049201	It can be lab-made
Cell Strainer-70µm	VWR	10199-656	
MCSF	Peprotech	315-02	
2-mercaptoethanol	Life Technologies	21985023	
Penicillin-Streptomycin (10,000 U/mL)	Life Technologies	15140122	
RPMI, Glutamax, HEPES	Invitrogen	72400-120	
50ml Conical Tube	VWR	21008-951	
Flow cytometer BD LSFRFortessa X-20	BD	656385	
23G needles	VWR	BD305145	
BD 3ML - SYRINGE	VWR	BD309657	Other syringes are acceptable too
Petri Dish 100mm x 15 mm	Fisher Scientific	F80875712	
Cell counter-Vi-CELL- XR Complete System	BECKMAN COULTER Life Sciences	731050	Cells can be manually counted too
Murine IL-4	Peprotech	214-14	
LPS-SM ultrapure (tlrl-smpls)5mg	Invivogen	tlrl-smlps	

Table 8. List of Seahorse related material (Continued).

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Seahorse XF Glycolytic Rate Assay Kit	Agilent Technologies	103344-100	
Seahorse XF 200mM Glutamine Solution	Agilent Technologies	103579-100	
XF RPMI Medium, pH 7.4 without phenol Red	Agilent Technologies	103336-100	
Seahorse XFe96 FluxPaks	Agilent Technologies	102416-100	
XF Glycolysis Stress Test Kit	Agilent Technologies	103020-100	
CyQUANT Cell Proliferation Assay Kit	Thermo Fisher Scientific	C7026	
XF Real Time ATP Rate Assay Kit	Agilent Technologies	103592-100	
XF Cell Mito Stress Test Kit	Agilent Technologies	103015-100	
XF Palmitate-BSA FAO Substrate	Agilent Technologies	102720-100	

ORO- Staining

In order to make foam cells, Day 7 BMDMs were starved in complete RPMI with 1% FBS. Then the cells in incubated with ox-LDL or LDL as a control. Cells were fixed with 4% PFA and stained with Oil Red O staining method (277). The lipid droplets in red color were detected and photographed with light microscopy.

The microscopic photography performed using Nikon eclipse TS 100 and processed with NIS Elements 4.0 software.

Statistical Analysis

Data were analyzed using GraphPad Prism Software 5.01. Comparisons between DMF and Indole were performed using a Student's t-test.

Sample analysis was performed using the T-test. data considered statistically significant as follow: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

One-way ANOVA, followed by Tukey's multiple comparison test, was applied to compare differences in experiments with multiple groups. data considered statistically significant as follow: at "*" $p < 0.05$, significance at "**" $p < 0.01$, significance at "***" $p < 0.001$, significance at "****" $p < 0.0001$.

Graphs

Graphical figures Created with BioRender.com. Or Microsoft PowerPoint. Other scientific graphs were created with GraphPad Prism V8.

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