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Delta Tocotrienol Attenuates NLRP3 Inflammasome Activation via Inhibition of NF-κB Priming and Reactive Oxygen Species Generation

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DELTA TOCOTRIENOL ATTENUATES NLRP3 INFLAMMASOME ACTIVATION VIA INHIBITION OF NF-κB PRIMING AND REACTIVE OXYGEN SPECIES GENERATION

by

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Chronic, low-grade inflammation during obesity is associated with the development of metabolic dysfunction. The NLRP3 inflammasome is assembled in response to cellular stressors and leads to cytotoxic cytokines IL-1β and IL-18 production, which implicates the NLRP3 inflammasome in inflammatory conditions, including type 2 diabetes. Tocotrienols are antioxidant and anti-inflammatory forms of vitamin E. Delta-tocotrienol (dT3) displays NF-κB inhibitory and anti-oxidant abilities, and is easily isolated from the Annatto plant. My primary aim was to determine whether dT3 inhibits NLRP3 inflammasome activation and to compare the extent to which dT3 inhibits NLRP3 inflammasome with other tocotrienol forms, i.e. alpha-tocotrienol (aT3) and gamma-tocotrienol (gT3). The second aim was to investigate the role of dT3 in NF-κB priming and reactive oxygen species (ROS) production, two major signaling pathways for NLRP3 inflammasome activation. To determine the inflammasome activation, J774 macrophages (iJ774) that stably overexpress an inflammasome reporter were pretreated with dT3, aT3, and gT3 and stimulated with lipopolysaccharide (LPS)/nigericin. Inflammasome activation was measured through Gaussia luciferase activity (iGLuc), IL-1β production, and caspase-1 and iGLuc immunoblotting. Raw 264.7 macrophages

pretreated with dT3, aT3, and gT3 were stimulated with LPS. We measured 1) gene expression of down-stream targets of NF-κB (*Nlrp3, Tnf-α, IL-1β, IL-18*) by qPCR and 2) degradation of inhibitory protein of NF-κB (IκBα) by Western blot. Raw 264.7 macrophages pretreated with dT3, aT3, and gT3 were stimulated with LPS/nigericin and ROS production was measured using an ROS-sensitive florescent probe. Results show that 1) 0.5-5 μM dT3 pretreatment inhibited NLRP3 inflammasome activation and IL-1β production; 2) 1 μM dT3, aT3, and gT3 pretreatment inhibited NLRP3 inflammasome activation and IL-1β production; 3) 1-2.5 μM dT3 inhibited NF-κB activation; 4) 1 μM dT3, aT3, and gT3 pretreatment diminished NF-κB activation in macrophages; and 5) 1 μM dT3, aT3, and gT3 decreased ROS production in LPS/nigericin treated macrophages. These studies demonstrated that tocotrienols (dT3, aT3, gT3) lowered NLRP3 inflammasome activation in macrophages through inhibition of NF-κB activation and ROS production with an apparent efficacy of gT3>dT3>aT3. It suggests that inclusion of dietary tocotrienols may constitute a novel dietary strategy to attenuate and/or prevent pathogenic progression of obesity.

DEDICATION

This work is dedicated to

My Mom, Kathy, and my Dad, Steve, for support and encouragement

Denise, Michael, Asher, and my 2 Pillars family

And my long-standing friend, coffee, who has always bean there for me during late nights

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CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Inflammation has traditionally been considered as swelling, fever, pain, and redness as a tissue response to injury (1). Inflammation can be beneficial during wound healing and infections, but low-grade chronic inflammation can wreak havoc on the body and its metabolic processes. Obesity, insulin resistance, and inflammation were first connected in 1901, when Williamson (2) found that the anti-inflammatory compound salicylate improved glucose control in diabetes. Since then, evidence has continued to accumulate that links metabolic disease with chronic low-grade inflammation. The effects of chronic inflammation during obesity are wide-spread. Obesity induces inflammatory changes in liver, muscle, the hypothalamus, pancreatic islets, and adipose tissue (3). Inflammation plays a role in overfeeding and energy expenditure (4). Inflammation is associated with atherogenesis and endothelial dysfunction (5,6), NASH (7) and liver fibrosis (8), and renal dysfunction (9). Inflammation may mediate the development of obesity-induced insulin resistance (10). Ameliorating inflammation has thus been therapeutically targeted for ameliorating the metabolic consequences of overweight and obesity and an unhealthy diet. Inflammation has also been targeted due to its central role in many chronic inflammatory diseases. It is indeed critical to determine the molecular mechanisms that connect obesity, inflammation, and metabolic dysfunction.

During chronic inflammation, there are many processes at work that lead to metabolic dysfunction. In the obese state, elevated levels of free fatty acids (FFAs),

lipopolysaccharide (LPS), peptidoglycan, and bacterial DNA among other signals initiate inflammatory gene transcription (11). Some theorize that the pro-inflammatory cytokines secreted from adipose lead to chronic inflammation and impairs both insulin signaling and mitochondrial function, and worsens cardiovascular disease risk (12). Endoplasmic reticulum (ER) stress, hypoxia, and lipotoxicity can be increased by obesity and lead to inflammation, and appear to be critical in the impairment of insulin resistance (11).

Inflammasomes are multimeric protein complexes studied for their role in inflammatory diseases and in mediating the complications of metabolic syndrome. There are many families of inflammasomes. They generally are comprised of a sensor molecule, ASC (apoptosis-associated speck-like protein containing a carboxy-terminal CARD) and caspase-1 (13). Inflammasomes detect danger signals through the sensor molecules. These signals include exogenous pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs). Upon signal detection, the sensor molecule triggers the ASC, which cleaves pro-caspase-1 to caspase-1 (13). The cleavage of caspase-1 is a key event in the activation of the inflammasome. Next, the inflammasome will be discussed—its mechanism of activation and effecting factors, its role in metabolic disease, and dietary factors modulating the inflammasome.

1.2 NLRP3 Inflammasome

The NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome is one of the more widely studied inflammasomes, particularly because of its wide range of activators. The amino-terminal death fold domain, central NACHT nucleotide binding domain, and carboxy-terminal leucine-rich repeats (LRRs) distinguish

the NLRP3 inflammasome from other inflammasomes (14). As with other inflammasomes, the NLRP3 inflammasome is signaled through DAMPs or PAMPs. NLRP3 inflammasome requires two steps: 1) a priming step that induces NLRP3, pro-IL-1β, and pro-IL-18 gene transcription, and 2) an activating step in which the inflammasome is assembled and caspase-1 is activated (13). This is completed through innate immune signaling or cytokine receptors (13).

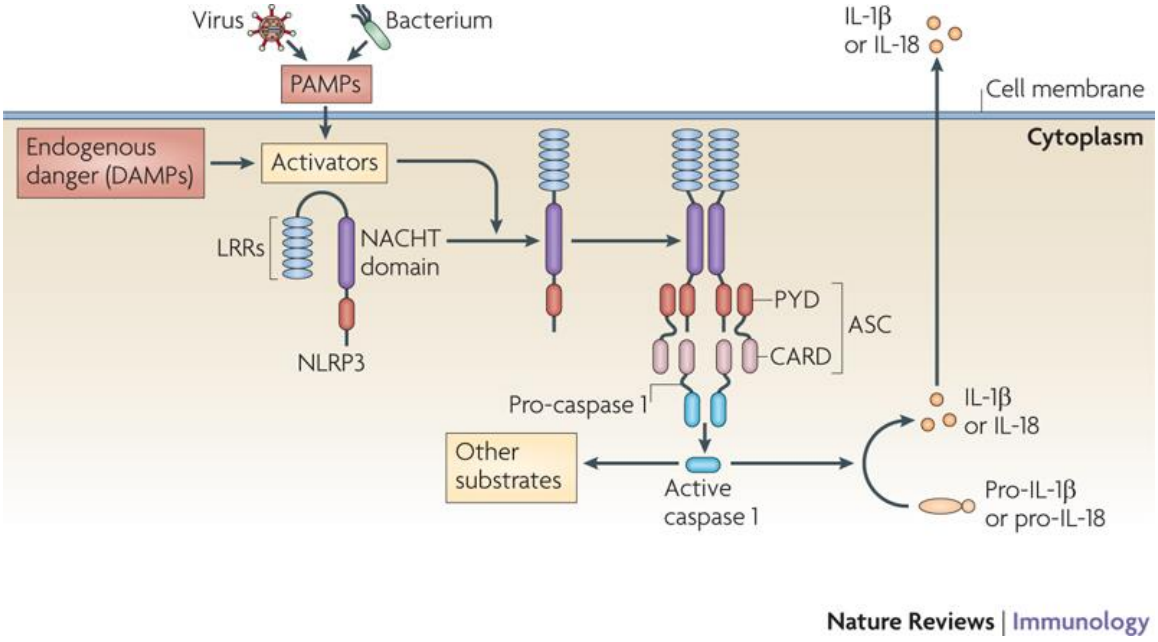


Figure 1. Structure and assembly of the NLRP3 inflammasome. In response to cellular stressors, the components of the NLRP3 inflammasome are assembled and activate caspase-1, which cleaves pro-inflammatory cytokines pro-IL-1β an pro-IL-18 to IL-1β and IL-18 (15).

Upon NLRP3 inflammasome activation, the cleaved caspase-1 will cleave the pro-inflammatory cytokines pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18) into their active forms, interleukin-1β (IL-1β) and interleukin-18 (IL-18). After release from the cell, these cytokines perpetuate the inflammatory signal and mediate the inflammatory response. IL-1β and IL-18 have far-reaching actions in the body, and are implicated in the development of acetominophin-induced hepatotoxicity, macrophage

migration in atherosclerosis, and the development of insulin resistance (8,16,17). The IL-1 family of cytokines, which includes IL-1β, are an essential mechanism of the innate immune system to protect against infections (18). These cytokines recruit macrophages and other immune cells to attack invading pathogens. IL-1β is a critical target for auto-inflammatory syndromes, and these diseases respond to blocking IL-1Ra, the IL-1 receptor antagonist (18). Auto-inflammatory conditions include such diseases as familial Mediterranean fever, cryopryin-associated periodic syndromes, Muckle-Wells syndrome, and neonatal onset multisystem inflammatory disease (18).

The NLRP3 inflammasome has functions beyond those carried out by IL-1β and IL-18 (19). NLRP3 inflammasome activation is also involved in pyroptosis, a type of cell death distinct from apoptosis (19). Besides cleavage of IL-1β, caspase-1 is involved in activating lipid biogenesis and inhibiting glycolysis during sepsis and pyroptosis (20-22). The inflammasome clears bacteria such as *Salmonella enterica* in an IL-1β- and IL-18-independent manner and contributes to infection control (19). Caspase-1 participates in the cleavage of so-called "leaderless proteins," such as high mobility group box 1 (HMGB1), a DAMP (19). Caspase-1 inhibits glycolysis by impairing the enzymatic ability of glyceraldeyde 3-phosphate during infectious invasions (19). Denes et al. (23) identified 121 substrates caspase-1 acts upon.

The NLRP3 inflammasome can be activated by a variety of exogenous and endogenous substances. Exogenous activators include asbestos (24), silica (24,25), maitotoxin (26), sendai virus, influenza virus, adenovirus, *Saccharomyces cerevisiae, Candida albicans, Shigella flexneri* (27), *Salmonella typhimurium* (28), *Escherichia coli* (29), *Staphylococcus aureus* (26,29), *Bacillus anthracis* (30), *Listeria monocytogenes*

(26), ricin toxin (31), and nigericin (26,32). Numerous endogenous activators include monosodium urate (MSU) (elevated in gouty arthritis) (27,33), calcium pyrophosphate dehydrate (elevated in calcium pyrophosphate deposition disease) (33), endogenous ATP (26,34), TNF-α (35), IL-1β (35), angiotensin-II (36), uric acid (27,34), high glucose (27), amyloid-β-fibrils (elevated in Alzheimer's disease) (27). Cytokines such as TNF-α and IL-1β that are secreted in response to the NLRP3 inflammasome also activate the inflammasome, and thus may participate in a positive feedback loop (35). Necrotic cells serve as an internal danger signal for the NLRP3 inflammasome (9). Due to its wide spectrum of activators, the NLRP3 inflammasome becomes active in a wide number of conditions.

NLRP3 inflammasome activation is involved in many auto-inflammatory conditions (35). Fibrosis involves the chronic activation of the NLRP3 inflammasome, which perpetuates damage through the synthesis of collagen and extracellular matrix protein (37). The NLRP3 inflammasome is implicated in pulmonary inflammatory diseases and links air pollutants to lung cancer and fibrosis (24), and silica-induced pulmonary fibrosis (25). Moreover, the NLRP3 inflammasome is involved in the development of cerebral malaria (38) and damage from UVB irradiation. The NLPR3 inflammasome provides the link between high glucose levels in metabolic (27) as well as amyloid-β-fibrils in Alzheimer's disease, and MSU and uric acid crystals in gout (39). Oslowski et al. (39) demonstrate that glucose toxicity and oxidative stress lead to endoplasmic reticulum (ER) stress and NLRP3 inflammasome activation. On the other hand, the NLRP3 inflammasome has beneficial activity during exercise. Acute exercise induces ROS production leading to NLRP3 activation and mitophagy to ameliorate

myocardial injury through clearance of damaged mitochondria and suppress further ROS-induced damage (40).

NLRP3 inflammasome is active in many different types of cells, including monocytes and macrophages (41), T cells (42), myofibroblasts (43), keratinocytes (44,45), and hepatic stellate cells (8). Lebeaupin et al. (46) demonstrated the NLRP3 inflammasome is active in non-myeloid cells, specifically hepatocytes. Unlike other cell types, blood monocytes constitutively express the NLRP3 inflammasome during the resting state, and require only one signal (18). Due to its large number of activators and cells in which it is active, the mechanisms of NLRP3 inflammasome activation are yet to be fully delineated.

1.3 NLRP3 Inflammasome Activation

The NLRP3 inflammasome assembly involves two steps: a priming step and an activating step (47,48). The priming step is necessary but not adequate to activate NLRP3 inflammasomes (49). This requirement for a priming step is another characteristic of the NLRP3 inflammasome that sets it apart from other inflammasomes (50). Pattern recognition receptors (PRRs) participate in NLRP3 inflammasome priming, in particular through toll-like-receptor (TLR) signaling (3,51-53). PRRs, including TLR4, are necessary for the development of insulin resistance due to high fat diet-(HFD) induced obesity (3). Priming the inflammasome increases gene expression of the cytokines, pro-IL-1β and pro-IL-18, as well as expression of NLRP3 inflammasome, and may be signaled through TLRs (26). TLRs activate nuclear factor κB (NF-κB) signaling (3) through TNF-α and TLR ligands to increase NLRP3 inflammasome activation (35).

NF-κB acts up-stream of ASC to increase NLRP3 inflammasome activation (49). LPS up-regulates inflammasome gene expression in the liver through TLR4 signaling (7). Extracellular and endosomal PAMPs, including LPS, signal through TLRs to increase pro- IL-1β synthesis (54). In the obese state, elevated free fatty acids, microbial LPS, peptidoglycan, bacterial DNA, and endogenous DAMPS signal Jun amino-terminal kinases JNK-AP1 and NF-κB through TLRs (11).

The second step is post-transcriptional and activates the assembly of the inflammasome (49). NLRP3 inflammasome assembly involves the protein thioredoxin interacting protein (TXNIP). Upon stimulation from substances such as uric acid crystals, TXNIP dissociates from thioredoxin (TRX) and binds to NLRP3 inflammasome allowing for activation (55). TXNIP participates in the glucose-induced secretion of IL-1β in β-cells (55). AMP-activated protein kinase (AMPK) phosphorylates TXNIP, which leads to a proteasome-dependent degradation of TXNIP, and down-regulates the binding of TXNIP to NLRP3 inflammasome (56). AMPK has been identified as a potent regulator of the pro-inflammatory inducible nitric-oxide synthase (iNOS), and could be a target for alleviating chronic inflammation in conditions such as obesity-induced diabetes (57). The exact mechanism of NLRP3 inflammasome activation is highly debated, and may involve multiple processes, including lysosomal damage and frustrated phagocytosis, intracellular K^+ efflux, ROS production, Ca^{2+} signaling, deubiquitination, and microRNA signaling.

A. Lysosomal Damage

One model that has been proposed is lysosome destabilization and frustrated phagocytosis (58). Crystalline and particle activators of the inflammasome seem to induce lysosome destabilization and cathepsin B secretion which triggers NLRP3 inflammasome (27). However, this model does not apply to the activators nigericin or R837 (27). Permeablization of the lysosome leads to deterioration of mitochondrial function and increases NLRP3 inflammasome activation (59). Heid et al. (59) also proposed that activation of the NLRP3 inflammasome involves damage to the mitochondria and lysosome which leads to inflammasome activation and cellular damage. Many other researchers have identified reactive oxygen species (ROS) as participating in inflammasome activation, and the induction of mitochondrial and lysosomal damage may link these mechanisms.

B. K^+ Efflux

Researchers implicate K^+ efflux in NLRP3 inflammasome activation (29). Franchi et al. (29) found that low intracellular K^+ is necessary for NLRP3 activation by *S. aureus* and *E. coli*. NLRP3 activation by silica was dependent on intracellular K^+ efflux as well as ROS production (25). Intracellular K^+ efflux is a factor in the activation of the inflammasome through extracellular ATP, MSU, and Nigericin (38). Petrilli et al. (60) and Shimada et al. (30) state that K^+ efflux is a unifying theme among NLRP3 inflammasome activators. However, Jin et al. (27) note that although intracellular K^+ efflux is common among many NLRP3 inflammasome activators, it may not be sufficient for activation. Extracellular K^+ decreases IL-1β secretion and NLRP3 inflammasome

activation in bone marrow-derived macrophages (BMDM) (59) and diminished mitochondrial damage in BMDM (28). Likewise, providing extracellular K^+ prevented K^+ efflux and inhibited caspase-1 activation in human monocytes (THP1 cells) (30). On the other hand, low intracellular K^+ triggers P2X purinoceptor 7 (P2X7R) to activate caspase-1 in macrophages (29). P2X7R binds with the NLRP3 inflammasome during activation (61). As discussed later, mitochondrial damage and ROS production play a role in inflammasome activation, and this could link these two common activation mechanisms.

C. ER Stress

The ER is considered a "nutrient-sensing" organelle (62). The metabolic stress of obesity can alter cell metabolism by increasing ER stress, mitochondrial dysfunction, which leads to ROS production and oxidative stress (62). The ER is highly sensitive to glucose metabolism and energy levels. ER stress may impair hepatic gluconeogenesis in obesity and type 2 diabetes (T2DM) (62). Hotamisligil et al. (62) postulate that ER stress is increased in metabolically active tissues during obesity. C-Jun N-terminal kinase-activator protein-1 (JNK-AP1) and NF-κB are two inflammatory pathways that are implicated in the development of insulin resistance, inducing oxidative and ER stress, and are signaled through saturated fatty acids (SFA) and are thus key to the relationship between obesity, insulin resistance, and inflammation (11). During ER stress, unfolded proteins accumulate, leading to the unfolded protein response (UPR) (62). The purpose is to decrease protein synthesis and increase protein degradation to relieve the stress of accumulated unfolded proteins (62). If ER stress is not resolved, it leads to apoptosis

(62). Cox et al. (63) demonstrated that ER stress leads to the unfolded protein response (UPR), which activates the transmembrane sensor inositol-requiring enzyme 1 (IRE-1α) to resolve the stress. Protein kinase R-like endoplasmic reticulum kinase (PERK) and IRE1-α, two kinases, and activating transcription factor 6 (ATF6) detect unfolded protein and activate UPR pathways (64). IRE1-α and PERK become engaged in response to ER stress, and ATF6 is diminished and signals to the Golgi apparatus (62). When IRE1 is engaged, it binds to mRNA to prevent protein translation (62). In addition to IRE1 signaling, ER stress also leads PERK to induce NF-κB activation, a transcription factor that increases pro-inflammatory gene expression (62).

ER stress occurs in NLRP3 inflammasome activation/assembly. Inhibiting ER stress through tauroursodeoxycholic acid (TUDCA) protected the livers of obese mice from LPS-induced injury, apoptosis, and inflammasome priming through decreasing IRE1-α and PERK activation (46). Decreasing ER stress through TUDCA prevented NLRP3 inflammasome activation in kidney epithelial cells (65). Angiotensin-II stimulation in human kidney-2 (HK-2) cells leads to ER stress which activates the NLRP3 inflammasome and increases caspase-1 activation and IL-1β and IL-18 secretion (36). Lerner et al. (64) found that ER stress acts post-transcriptionally to increase IL-1β b secretion. Contrarily, Kim et al. (66) found that ER stress both increased NF-κB activation (which increased pro-IL-1β expression) and induced IL-1β secretion in BMDM.

Insulin resistance can lead to ER stress and is related to PERK and JNK signaling (62). In pancreatic β-cells, PERK and IRE1-α signal TXNIP, ultimately inducing NLRP3 inflammasome activation and IL-1β secretion (39). ER stress signals the inflammasome

through dissociating dissociates TXNIP from TRX (67). Lerner et al. (64) found that UPR induces TXNIP through IRE1-α. IRE1-α induces TXNIP activity through the microRNA miR-17, and TXNIP activates NLRP3 inflammasome and increases IL-1β secretion through increased levels of ROS (64). Lerner et al. (64) demonstrated the TXNIP activation of NLRP3 inflammasome mediated β-cell death due to ER stress, leading to diabetes in rodents. On the other hand, Lebeaupin et al. (46) showed that PERK and IRE1-α activate C/EBP homologous protein (CHOP), which in turn, acts on the NLRP3 inflammasome and hepatic apoptosis. In the hepatocytes, TXNIP does not appear to play a role in ER-stress induced inflammasome activation, though the IRE1-α - PERK-CHOP axis is pivotal to ER-mediated inflammasome damage in the liver (46).

Zhao et al. (67) connected ER stress to ROS since the increase in protein folding also increases ROS production in the ER. ROS production and TXNIP was essential to NLRP3 assembly and activation in macrophages (66). Decreasing ER stress through increased AMPK activity and decreased IRE1-α and PERK phosphorylation decreased intracellular ROS and inhibited NLRP3 inflammasome activation (68). Lowering IRE1-α phosphorylation ameliorates ER stress as well as ROS production in response to high glucose in endothelial cells (69). Menu et al. (70) found that both K^+ efflux and ROS production are necessary when the NLRP3 inflammasome is activated through ER stress. Blocking K^+ efflux and ROS production in human macrophages inhibited ER-stress induced IL-1β secretion (70). The researchers also proposed that neither phagocytosis nor UPR was required for NLPR3 activation. This demonstrates that the role of UPR and ER stress may be specific to cell type (58).

D. ROS

Many researchers suggest that many NLRP3 inflammasome activators lead to ROS production (27). For example, extracellular ATP increases ROS production and activates the NLRP3 inflammasome in macrophages (71). Some argue that ROS is necessary for NLRP3 inflammasome activation because ROS inhibitors inhibit NLRP3 inflammasome activation (24). Indeed, inhibiting ROS production inhibits both NLRP3 inflammasome expression and activation (25,72), and IL-1β production (59). Inhibiting mitochondrial ROS through N-acetyl-L-cysteine (NAC) lowered expression of NLRP3 and IL-1β and IL-18 (73). Scavenging mitochondrial ROS through molecular hydrogen, which targets the mitochondria and scavenges ●OH, inhibited NLRP inflammasome in mouse macrophages (74). Li et al. (40) demonstrated that during acute exercise, increased oxidative phosphorylation increased ROS production and led to NLRP3 inflammasome activation and mitophagy in the myocardiam. Bauernfeind et al. (50) found that inhibiting ROS blocked the priming step of NLRP3, not the activating signal. Changes in the redox balance in cells towards a more reduced state during chronic disease may activate the innate immune response to activate the NLRP3 inflammasome and produce IL-1β (75). The NLRP3 inflammasome is particularly sensitive to changes in redox conditions because of a highly conserved disulfide bond in the PYD domain and the nucleotide-binding site domain (76). During the resting state, monocytes have a balanced redox state, but TLR signaling leads to an oxidative hit which increases ROS production that activates NLRP3 inflammasome and produces IL-1β (75). Carta et al. (75) proposed that the difference in redox states between THP-1, monocytes, and raw macrophages accounts for differing IL-1β responses to pro-oxidants and antioxidants.

Indeed, ROS oxidize TRX and release TXNIP, which binds to the NLRP3 inflammasome (55).

The source of the ROS that activates the NLRP3 inflammasome is not yet clear. It was first hypothesized that NADPH oxidases were the source of the ROS that activate NLRP3 inflammasome (24). Liao et al. (77) found that ROS production through NADPH oxidase was required for priming and activation stages of NLRP3 inflammasome activation in mouse macrophages. LPS increased NF-κB activation through NADPH oxidase ROS, and ATP induced NLRP3 inflammasome assembly through NADPH oxidase in macrophages (77). Endogenous ROS production that was NADPH oxidase-dependent in mouse glomeruli activates the NLRP3 inflammasome and perpetuates hyperhomocysteinemia (78). Similarly, NADPH oxidase was found to play a role in NLRP3 inflammasome activation in mouse podocytes and in the recruitment of macrophages and T-cells, as well as the development of hyperhomocysteinemia (79). Artlett et al. (37) note that ROS production from NADPH oxidase is involved in the chronic activation of NLRP3 inflammasome in fibrotic diseases. Despite the evidence implicating NADPH oxidase in NLRP3 inflammasome activation, both mouse and human models lacking NADPH oxidase retained NLRP3 inflammasome function (80-84).

Mitochondrial dysfunction appears to be involved in ROS-mediated NLRP3 inflammasome activation as well. NLRP3 inflammasome activation impairs mitochondrial function (59). Inhibiting mitochondrial ROS lowers IL-1β secretion (59). Inducing ROS production and mitochondrial stress activates the NLRP3 inflammasome and causes cell pyropotsis and apoptosis in HaCaT cells (85). Kim et al. (66) found that a

reduction in mitochondrial membrane potential induced ROS production that activated NLRP3 inflammasome. Mitochondrial ROS production increases NLRP3 inflammasome activity and IL-1β production in macrophages (86). Scavenging mitochondrial ROS through molecular hydrogen inhibited NLRP inflammasome in mouse macrophages (74). Down-regulation of the NLRP3 inflammasome protected the hippocampus from ischemic-related damage and preserved mitochondrial function from glutamate neurotoxin (68).

The decrease in mitochondrial ROS decreased NLRP3 inflammasome activation through ATP stimulation (87). Oh et al. (87) found that inhibiting mitochondrial ROS production in macrophages did not affect transcriptional levels of NLRP3 or pro-IL-1β, but diminished NLRP3 inflammasome activation. At rest, the NLRP3 inflammasome is mostly located at the mitochondria-associated ER membrane (MAM), and upon stimulation, ASC relocates to the MAM. This relocation is dependent on the presence of NLRP3 (88). Additionally, ROS production caused TXNIP to relocate to the mitochondria (88). The mitochondrial damage that resulted in ROS production was due to inhibition of autophagy and mitophagy (88). This was corroborated by Nakahira et al. (89), who demonstrated that depleting autophagic proteins impairs mitochondrial integrity and increases ROS production, which activates the NLRP3 inflammasome. Sorbara et al. (90) suggest that NLRP3 inflammasomes are activated by mitochondrial ROS, and autophagy clears dysfunctional mitochondria leading to inhibition of the NLRP3 inflammasome.

Nakahira et al. (91) also found that NLRP3 inflammasome activation results in release of mitochondrial DNA (mtDNA). Molecular hydrogen inhibits mtDNA priming

of NLRP3 inflammasome (74). Additionally, blocking NLRP3 inflammasome activation simultaneously decreases mtDNA release (72). Activation through ATP induces mtDNA release to the cytosol, which binds to and activates the NLRP3 inflammasome (28). Bauernfeind et al. (50) hypothesize that the differential requirements for ROS production in inflammasome activation are due to the fact that cells express varying levels of NLRP3 inflammasome, and thus may or may not need to be primed. Menu et al. (70) found that dampening mitochondrial activity inhibited ER-stress induced NLRP3 inflammasome activation, but not when human macrophages were stimulated with MSU. This indicates that the involvement of mitochondrial ROS may depend on the stimulant.

In addition to NADPH oxidase and mitochondrial dysfunction, there are other sources of ROS, including cyclooxygenases (COXs), cytochrome P450s, lipoxygenases (LOXs), and xanthine/xanthine oxidase (X/XO) (58). These sources of ROS have the potential to affect the inflammasome, but their role has yet to be fully elucidated. Inhibiting ROS production diminished NLRP3 activation through the mitigation of oxidized LDL (oxLDL), but the authors suggested that K^+ efflux and lysosome destabilization may play a role as well (92). Wu et al. (91) suggested that ROS production due to palmitic acid addition links ER stress with TXNIP induction and NLRP3 inflammasome activation.

E. Intracellular Ca^{2+}

Ca^{2+} signaling appears to be involved with NLRP3 inflammasome activation as well. Ca^{2+} increases during cell stress, which lowers cAMP, ultimately activating the inflammasome (93). Artlett et al. (37) suggest modulating calcium homeostasis may

regulate inflammasome activation. Ca^{2+} plays a role in inflammasome activation by ATP, nigericin, MSU, and alum (94). Murakami et al. (94) found that inhibiting mobilization of Ca^{2+} stores prevented ATP-induced NLRP3 inflammasome activation. During ATP-stimulated inflammasome activation, Ca^{2+} is released from the ER leading to an influx of extracellular Ca^{2+} (94). The transcription factor CCAAT-enhancer-binding protein (C/EPB) can release Ca^{2+} from the ER and lead to NLRP3 inflammasome activation, which shows how ER stress may activate the inflammasome (94). Phaygolysosomal rupture may lead to Ca^{2+} influx because the lysosomotropic peptide Leu-Leu-OMe induces both phagolysosomal rupture and the NLRP3 inflammasome, and lysosome rupture releases stored Ca^{2+} (94). Flux of Ca^{2+} also leads to mitochondrial damage (94). Murakami et al. (94) demonstrate that K^{+} efflux may lead to Ca^{2+} influx, and sustained Ca^{2+} influx can damage the mitochondria, which could lead to inflammasome activation. Inhibiting Ca^{2+} signaling blocked mtROS production as well (94). Chelating calcium protected from oxidative damage, and this demonstrated that calcium flux leads to ROS production (95). However, Ca^{2+} influx is not sufficient for NLRP3 inflammasome activation (94).

F. Additional Mechanisms

Deubiquitination and microRNAs have also been proposed as other mechanisms of NLRP3 inflammasome activation (58). NLRP3 inflammasome activation through deubiquitination is due to a basal level of NLRP3 that is ubiquitinated but can be deubiquitinated upon LPS or ATP treatment, which signal activation through TLRs and mtROS in mouse macrophages (96). MicroRNAs (miR) may also impact NLRP3

inflammasome activation. MiR-223 has been specifically identified as a potential regulator of the NLRP3 inflammasome (97,98). High levels of miR-223 lowers NLRP3 protein levels and decreases IL-1β secretion, and infecting B cells with exosomes containing miR-223 inhibited NLRP3 inflammasome activation (98).

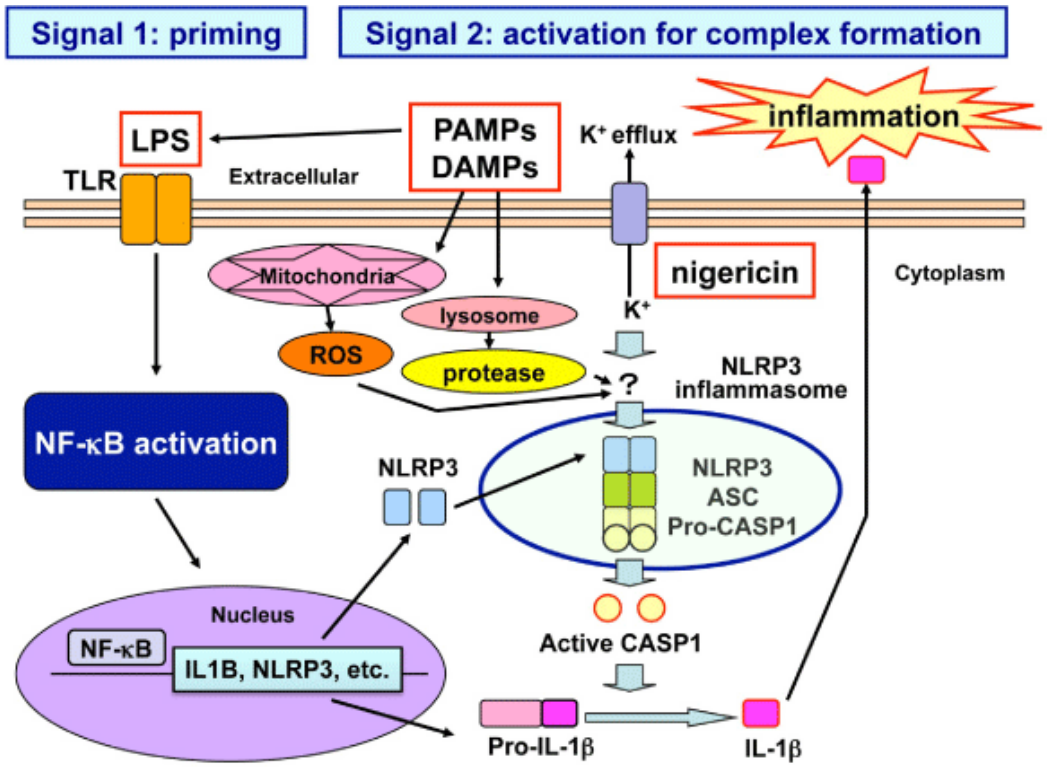


Figure 2. A summary of NLRP3 inflammasome activation. NLRP3 activation requires two signals—a priming signal through NF-κB, and an activating signal. The second signal involves ROS production, K^+ efflux, lysosomal degradation, Ca^{2+} signaling, and ER stress. (99)

1.4. Metabolic Implications of NLRP3 Inflammasome Activation

A. The Relevance of IL-1β Production in NLRP3 Inflammasome

IL-1β is a pro-inflammatory cytokine that is linked with many inflammatory conditions. IL-1β is implicated in cognitive decline (100). IL-1β is also implicated along with cyclooxygenase-1-mediated prostaglandins in the development of acute cognitive deficits (101). Importantly, it may lead to the development of insulin resistance. A study

based on the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam study, which included 27,548 individuals found that plasma levels of IL-1β and IL-6 were predictive of the risk of T2DM (102). In obese patients, IL-1β and IL-1Ra are up-regulated in adipose, demonstrating its integral role in the pathogenesis of obesity-related diseases (103). In addition, higher levels of IL-1β and IL-1Ra affected the hypothalamus and adipocyte metabolism resulting in weight gain (103).

High glucose concentration in pancreatic β-cells leads to IL-1β production and NF-κB activation, which led to β-cell death (104). In β-cells, IL-1β leads to iNOS gene expression, which produces nitric oxide (NO), ultimately contributing to β-cell death and insulin resistance (105). NF-κB increases gene expression of iNOS, which, together with IL-1β, mediates β-cell death (106). IL-1β increased NF-κB expression, and NF-κB activation induced iNOS and monocyte chemoattractant protein-1 (MCP-1) (107). Khodabandehloo et al. (108) demonstrated that IL-1β and TNF-α activate iNOS which leads to Akt inactivation and subsequent impairment of insulin sensitivity. IL-1β is also related to increased insulin sensitivity since IL-1β induces ER stress through Ca^{2+} flux, which leads to β-cell death (109). In C57BL/6 mice treated with IL-1β antibodies for 13 weeks demonstrated improved hemoglobin A1c (HbA1c), decreased insulin levels, and improved blood glucose (110). Anakinra, a well-documented IL-1Ra antagonist, lowers HbA1c and fasting glucose in type 2 diabetic patients (111). Though Larsen et al. (112) confirmed that IL-1Ra antagonists improved HbA1c and blood glucose levels, there was not a reciprocal improvement in insulin resistance. IL-1β secreted from NLRP3 inflammasome activation is thus an important target for improving outcomes of inflammatory conditions.

B. Obesity

The NLRP3 inflammasome seems to be implicated in the pathophysiology of obesity. Vandanmagsar et al. (17) found that the presence of NLRP3 is key for activation of inflammation in adipose and liver by obesity. These researchers demonstrated that NLRP3 inflammasomes detect danger signals that lead to obesity-related inflammation and insulin resistance (17). As body weight and adiposity increases, IL-1β and NLRP3 mRNA levels increase in the visceral adipose tissue (VAT) (17). Activation of the NLRP3 inflammasome in obesity leads to IL-1β and IL-18 production (17). NLRP3 ablation lowered interferon-γ (IFN-γ) and IL-18 levels and T cells in the adipose (17).

Adipose tissue is particularly affected by NLRP3 inflammasome activation during obesity. Both caspase-1 and NLRP3 inflammasome activation affect adipocyte formation (113). During inflammation, anti-inflammatory macrophages can be polarized into a pro-inflammatory state. Deletion of the NLRP3 inflammasome mitigated adipose tissue macrophage (ATM) pro-inflammatory polarization in obesity (17). NLRP3 knockout mice were protected from HFD induced obesity and demonstrated a lower adipocyte size (4). NLRP3 knockout altered plasma leptin and resistin, affecting energy use, showing that NLRP3 inflammasomes modulates energy expenditure in overfeeding (4). Mice deficient in caspase-1 fed a HFD expended greater energy compared to wild type mice fed a HFD, though total energy intake was the same (4). The NLRP3 inflammasome can become activated during obesity, and helps perpetuate the detrimental effects of excess weight. This activation of the inflammasome during obesity is related to metabolic dysfunction, such as cardiovascular disease, diabetes, and liver disease.

C. Cardiovascular Disease

The inflammasome is implicated in the development of cardiovascular disease. Deletion of ASC improved parameters related to pulmonary hypertension in mice (114). Liu et al. (115) found that caspase-1 and NLRP3 inflammasome activity in the aorta was associated with vascular dysfunction in rats. NLRP3 inflammasome expression is significantly higher in patients that are obese, or have dyslipidemia, diabetes, or hyperuricemia (116). Additionally, coronary artery disease and coronary atherosclerosis were positively correlated with NLRP3 inflammasome expression in human subcutaneous adipose tissue (116). Patients with acute coronary syndrome demonstrate higher levels of the NLRP3 inflammasome in peripheral blood monocytes (117).

NLRP3 inflammasome activation is associated with impaired endothelial dysfunction, measured through the vasodilator response (5). Cholesterol crystals, the result of hypercholesterolemia, activate the NLRP3 inflammasome and lead to the down-stream production of HMGB1, which damages endothelial tissue (5). HMGB1 is an early event during atherogenesis and endothelial dysfunction (5). Fearon et al. (6) found that NLRP3 inflammasome and IL-1β production is implicated in the development of atherogenesis in both mice and humans. The inflammasome is further implicated in atherosclerosis, since P2X7R and NLRP3 expression is increased in atherosclerotic plaque (118). oxLDL further increases the expression of these pro-inflammatory proteins (118). In addition, knockdown of P2X7R, which binds to NLRP3 inflammasomes during activation, protected mice from atherosclerosis development (118). The role of the inflammasome in atherosclerosis is not fully delineated, but oxLDL may activate the

NLRP3 inflammasome, and the IL-1β secretion leads to foam cell formation (92). Within plaque, oxLDL activates NLRP3 inflammasome activation and IL-1β production, and this increased inflammation destabilizes the plaque (119). Inflammasome activation may therefore worsen atherosclerosis in the context of hypercholesterolemia (58).

Cholesterol crystals, elevated from hypercholesterolemia, activate the NLRP3 inflammasome in coronary arteries (5). Activation of the NLRP3 inflammasome from cholesterol crystals may be mediated through superoxide production (5). Duewell et al. (120) also found that cholesterol cholesterols activated the NLRP3 inflammasome. In atherosclerosis-prone Apo-E deficient mice, cholesterol crystals induced IL-1β release, but this was eliminated in NLRP3- and ASC- deficient macrophages (120). Duewell et al. (120) propose that oxLDL could provide the priming and the activating signal, releasing IL-1β. These researchers demonstrate that NLRP3 inflammasome activation in bone marrow derived cells promoted atherosclerosis development in mice (120).

D. Diabetes

Metabolic inflammation during obesity seems to be critical to developing insulin resistance, and is a unifying factor in processes including ER stress, hypoxia, and lipotoxicity (11). During obesity, increased inflammation is seen in tissues such as the liver and pancreatic islets, which can induce metabolic dysfunction (11). NLRP3 inflammasome activation impairs glucose homeostasis and insulin signaling (121). Vandanmasgar et al. (17) demonstrated that the NLRP3 inflammasome is critical for the development of peripheral insulin resistance and T2DM through the action of IL-1β.

NLRP3 inflammasome impairs insulin sensitivity (113), and NLRP3 ablation improved insulin signaling (17).

The NLRP3 inflammasome is critical for the development of T2DM from islet amyloid polypeptide (IAPP) activation, which forms in the pancreas during T2DM (122). In addition, activation of the JNK-AP1 and NF-κB pathways interferes with insulin sensitivity by increasing inflammatory cytokines and decreased GLUT4 expression (11). NLRP3 deficient mice had lower glucose levels and improved insulin sensitivity, which was linked to mitogen-activated protein kinases (MAPK) pathway activation in visceral fat (17). SFA priming of the inflammasome links HFD-induced obesity to insulin resistance and inflammation through altering cytokine production in dendritic cells (123). This could be due to the production of cytokines, since IL-1β impairs insulin signaling in vitro (121). In obese type 2 diabetic patients, decreased NLRP3 expression in adipose tissue is associated with lowered inflammation and improved insulin signaling (17). NLRP3 knockout protected mice from obesity-induced insulin resistance (4). In mice fed a HFD for 16 weeks, the deletion of TLR4 signaling in hematopoietic cells protected from fasting hyperinsulinemia, which displayed improved insulin sensitivity despite being obese (10). This demonstrates the critical role of TLR4 signaling in the development of HFD-induced insulin resistance (10). Taken together, this evidence shows that the inflammasome is important for linking obesity, inflammation, and T2DM.

E. Liver Disease

Liver damage involves NLRP3 inflammasome activation. The NLRP3 inflammasome can be activated in hepatocytes (46), and it is implicated in alcohol-

induced liver inflammation, liver damage, and steatosis (34). Alcohol damage to hepatocytes induces the release of ATP and soluble uric acid which leads to the secretion of IL-1β from liver immune cells (34). The NLRP3 inflammasome mediates liver damage due to acute alcohol binge-drinking (34). Additionally, NLRP3 inflammasome derived IL-1β and IL-18 leads to hepatotoxicity induced by APAP (acetaminophen) (124). The NLRP3 inflammasome has additionally been linked with hepatic steatosis. NLRP3 inflammasome expression is increased in the livers of patients with NASH and chronic hepatitis C virus (7). In contrast, ablation of NLRP3 improves hepatic steatosis (17). Similarly, NLRP3 knockout in mice led to lower hepatic triglyceride content (4). The NLRP3 inflammasome is further implicated in the development in liver disease, because it is required for liver fibrosis development (8). In the liver, the NLRP3 inflammasome plays a role in inflammation and the development of many liver diseases.

F. Renal Disease

The NLRP3 inflammasome also plays a role in kidney disease. It is activated in the kidneys of diabetic patients and is involved in proteinuria (65). The NLRP3 inflammasome is also involved in albuminuria (73). Nondiabetic patients with acute and chronic kidney disease (i.e. IgA nephropathy, minimal change disease, membranous glomerulopathy, lupus nephritis, secondary focal segmental glomerulosclerosis (FSGS), and hypertension/vascular nephrosclerosis) have increased gene expression of NLRP3 that correlates to the level of kidney function (125). In addition, inflammasome activation may mediate tubulointerstitial fibrosis in the renal tubule (65). The NLRP3 inflammasome plays a role in renal dysfunction and the influx of neutrophils during renal

ischemic acute tubular necrosis (9). Caspase-1 activation may trigger pyroptosis, which may play a role in the damage from glomerular sclerosis and end-stage renal disease (ESRD) (58). NLRP3 inflammasome activation is implicated in hyperhomocysteinemia (78). Xia et al. (126) found that high levels of plasma homocysteine activates the NLRP3 inflammasome in mouse glomeruli. NLRP3 ablation ameliorated hyperhomocysteinemia-induced glomerular and podocyte injury (126). Nlrp3 gene deletion protected from unilateral ureteral obstruction induced inflammation, fibrosis, and tubular injury (125). NLRP3 inflammasome activation in the kidney can promote inflammatory-related damage and kidney disease.

1.5 Dietary Regulators of the NLRP3 Inflammasome

A. Fatty Acids as Regulators of the NLRP3 Inflammasome

It is well known that diets high in saturated fats may be detrimental to health. Mice fed a long-term HFD (9 months) demonstrated NLRP3 activation in the liver (7). The relationship between the NLRP3 inflammasome and dietary fat may partially explain the metabolic consequences of dietary fat. Snodgrass et al. (127) demonstrated that dietary fat affects IL-1β secretion from blood monocytes. Many have studied the effect of a HFD on adipocyte inflammation. These studies reveal that 1) in mice, a HFD primed adipose tissue to produce IL-1β in vivo (128). 2) HFD in mice increases adiposity and caspase-1 activation in adipose (17). HFD increases caspase-1 expression, as well as IL-1β and IL-18 production levels (113). This increase in cytokines may lead to metabolic dysfunction and an impaired glucose handling. The development of insulin resistance due to a HFD is well-characterized in adipose tissue. Bone marrow-derived macrophages

(BMDCs) from HFD fed mice impaired insulin sensitivity in 3T3L1 adipocytes compared to BMDCs from chow-fed mice (123). HFD primed BMDC cross-talk with adipocytes, resulting in diminished insulin signaling (123). HFD increased expression of TLR4, caspase-1, and NLRP3 in BMDCs (123). This may explain the higher reactivity of these HFD-derived BMDCs to LPS and ATP inflammasome activation (123). It appears that a HFD increases inflammation through NLRP3 inflammasome activation, and the types of fatty acids in the diet differentially affect inflammasome activation.

a. Saturated Fatty Acids

High intake of SFAs is known to be detrimental to health, and this correlation holds true with NLRP3 inflammasome activation. SFA priming of the NLRP3 inflammasome impairs insulin sensitivity and leads to adipose inflammation in HFD-induced obesity (123). SFA-high diets signal NLRP3 inflammasome priming, leading to an inflammatory BMDC phenotype and impaired insulin signaling (123). Palmitate activates the NLRP3 inflammasome in many cell types, including hepatocytes (7), human monocytes (127), endothelial cells (67), primary macrophages, monocytic leukemia cells, differentiated adipocytes (66), and hematopoietic cells (121). In hepatocytes, palmitic acid activates the NLRP3 inflammasome and increases LPS sensitivity (7). Palmitate activates NLRP3 inflammasome-related IL-1β production (129). Inflammasome activation by palmitate in hepatocytes lead to danger signals that produce TNF-α production in liver mononuclear cells (7). Treatment with palmitate leading to inflammasome activation and IL-1β secretion impaired insulin action in vitro (121).

There are several mechanisms by which palmitate increases NLRP3 inflammasome activation. Palmitic acid activated NLRP3 inflammasome in raw 264.7 macrophages through TLR2 and TLR4 signaling that activated JNK and IκB kinase (IKK) cascades (130). Snodgrass et al. (127) found that palmitic acid increases NLRP3 inflammasome through TLR2 signaling, which increase IL-1β production in human monocytes. TLR2 signaling induced NF-κB activation (127). Monocytes express NLRP3 inflammasome and caspase-1 constitutively during the steady state, and stimulation with palmitate increased NLRP3 expression and IL-1β secretion (127). In the endothelium, palmitic acid increases phosphorylation of IRE1-α and ROS production through ER stress and oxidative stress (67). Palmitate induced IL-1β production in primary macrophages, monocytic leukemia cells, and differentiated adipocytes through induction of ER stress (66). Wen et al. (121) propose that palmitate activates the NLRP3 inflammasome through ROS production and K^+ efflux which led to increased IL-1β and IL-18 secretion in hematopoietic cells and macrophages. Palmitate inhibits AMPK, which lowered autophagy and increased mitochondrial ROS, leading to NLRP3 inflammasome activation (121).

Stearate also induces NLRP3 inflammasome in human monocytes/macrophages (131). Both stearate and palmitate increased IL-1β production and NLRP3 activation in THP1 cells (131). Nguyen et al. (130) found that arachidonic was the most potent FFA that activates the NLRP3 inflammasome, and myristic acid was unable to activate the inflammasome. More controversial is ceramide's role in inflammasome activation. Diets high in saturated fat can increase ceramide synthesis levels (132). Ceramide levels increase in adipose and plasma in C57BL/6J mice fed a HFD (133). Ceramides activate

the NLRP3 inflammasome in BMDMs (17). Adipose tissue macrophages (ATMs) stimulated with ceramides had increased caspase-1 activation, though this did not occur in NLRP3 knockout ATMs from mice (17). Alterations in ceramide metabolism may contribute to metabolic syndrome (12). Although Camell et al. (129) demonstrated that ceramide can activate the NLRP3 inflammasome through ROS production, mice lacking ceramide de novo synthesis in macrophages still demonstrated insulin resistance, leading to the conclusion that this pathway is not necessary for NLRP3 inflammasome activation. Ceramide synthesis may be dispensable during inflammation related to diet-induced obesity (129). Taken together, these studies show that a high intake of SFAs can lead to NLRP3 inflammasome activation and the development of metabolic dysfunction due to the increase of inflammation.

b. Unsaturated Fatty Acids

In contrast to SFA, Wen et al. (121) found that the unsaturated fatty acid (UFA) oleate does not activate the NLRP3 inflammasome. Csak et al. (7) demonstrated oleic acid and linoleic acid do not increase NLRP3 gene expression in hepatocytes and macrophages. Linoleic acid, a polyunsaturated fatty acid (PUFA), does not prime the NLRP3 inflammasome and increase pro- IL-1β levels (123). Moreover, the UFAs oleate and linoleate decreased IL-1β secretion in THP-1 cells stimulated by SFAs, and also Nigericin, alum, and MSU (131). The addition of UFAs (oleate and linoleate) decreased IL-1β production, but not its transcription, whereas SFAs increased IκBα and P65 phosphorylation (131). Monounsaturated fatty acid (MUFA)-HFD impaired NLRP3 inflammasome-mediated IL-1β production in pre-primed cells (128). MUFA-HFD also

ameliorated insulin resistance and hyperinsulinemia in obese mice (128). C57BL/6 mice on a diet supplemented with 3% menhaden fish oil and 7% safflower oil had decreased mRNA expression of caspase-1, NLRP3, and IL-1β compared to an isocaloric diet with10% safflower oil (134). UFAs may inhibit NLPR3 inflammasome through induction of AMPK. The MUFA oleic acid impaired IL-1β secretion in ATP-primed cells through up-regulation of AMPK (128). MUFA-HFD in mice lowered pro- IL-1β levels, IL-1β production, increased AMPK activation, and ultimately improved insulin sensitivity compared to SFA-HFD in mice (128). These studies support the widely accepted view that dietary UFAs are beneficial.

c. Omega-3 Fatty Acids

Omega-3 fatty acids are known to mitigate inflammation. This anti-inflammatory power may be in part due to inhibition of NLRP3 inflammasome. Docosahexaenoic acid (DHA), an omega-3 fatty acid, lowered pro-IL-1β expression induced by palmitic acid in human monocytes (127). Yan et al. (135) found that omega-3 fatty acids, specifically DHA and eicosapentaenoic acid (EPA) inhibited NLRP3 inflammasome. These researchers then investigated potential anti-inflammatory mechanisms that could be at work. They found that enzyme modified enzymatic products, such as Protectin D1 (PD1), Resolvin D1 (RvD1) and aspirin-triggered Resolvin D1 (ATRvD1), by lipoxygenases (AlOX5 and AlOX15) and aspirin-acetylated COX-2 were not involved (135). Instead, they found G-protein coupled receptor (GPR) 40 and 120 were at work (135). DHA and EPA signaled GPR40 and GPR120 (135). This signaling was required for the inhibitory effect of omega-3 FA on inflammasome activation (135). GPR40 and

GPR120 signaled overexpressed Arrestin, Beta 2 (ARRB2) to bind the LRR and NACHT portions of NLRP3 (135). Williams-Bey et al. (136) also investigated the mechanism behind DHA's and EPA's inhibitory effect on the NLRP3 inflammasome. These researchers found that DHA and EPA acted on the priming step of NLRP3 inflammasome activation (136). Omega-3 fatty acid treatment prevented nuclear translocation of NF-κB in THP-1 and mouse BMDMs (136). Omega-3s increased intracellular calcium and increased the interaction between β-arrestins and free fatty acid receptor 4 (FFAR4), which ultimately down-regulated NF-κB and Jun kinase (136). In addition, DHA increased autophagy in macrophages, which also decreased inflammasome activation because the NLRP3 inflammasome is degraded by the autophagosome (136).

DHA supplementation lowered fasting glucose, improved insulin sensitivity and glucose sensitivity in an NLRP3-dependent manner in mice fed a HFD (135). DHA also lowered production of pro-inflammatory cytokines, though this was not seen in NLRP3 knockout mice (135). This demonstrated that omega-3 fatty acids exhibit anti-inflammatory effects that improve type 2 diabetic complications through GPR40 and GPR120 regulation of the NLRP3 inflammasome (135). Reynolds et al. (137) notably found that in rats, male, but not female, offspring from HFD-fed mothers had increased expression of NLRP3, IL-1β, and TNF-α. This trend was reversed with conjugated linoleic acid (CLA) supplementation (137). Omega-3 fatty acids appear to be beneficial in inhibiting NLRP3 inflammasome activation.

B. Dietary Bioactive Compounds as Regulators of the NLRP3 inflammasome

Polyphenols are dietary bioactive compounds that have many beneficial actions in the body. Certain polyphenols—curcumin, quercetin, resveratrol, and epigallocatechin-3-gallate (EGCG)—have been demonstrated to decrease NLRP3 inflammasome activation. Curcumin inhibits NLRP3 inflammasome activation and caspase-1 levels in macrophages treated with LPS (138). Curcumin inhibited K^+ efflux, which limited IL-1β production and HMGB-1 levels, which is a DAMP (138). Quercetin, luteolin and epigallocatechin gallate are all flavonoids that increase AMPK phosphorylation (91). Treatment with these compounds also lowered ROS production which inhibited TXNIP and NLRP3 inflammasome activation when stimulated with palmitate (91). These compounds inhibited mitochondrial cell apoptosis, mitigated inflammation in endothelial cells, and reduced cell death (91).

Quercetin, a dietary flavonoid inhibits ROS production and hepatic oxidative stress in streptozotocin (STZ)-induced diabetic rats (139). Wang et al. (139) found that 50 and 100 mg/kg body wt quercetin (administrated through intragastric gavage) diminished TXNIP levels, as well as NLRP3 inflammasome activation and IL-1β secretion in the livers of STZ-injected diabetic rats and in HepG2 and BRL-3A cells treated with high glucose levels. Wu et al. (91) demonstrated that in endothelial cells, 10 μM quercetin increases AMPK phosphorylation and decreases ROS levels and TXNIP. This decrease in ROS and increase in AMPK phosphorylation led to lowered NRLP3 inflammasome activation and lowered gene expression of IL-1β (91).

Resveratrol is a polyphenol that is most well-known for its presence in wine. Supplementing mice fed a HFD with resveratrol improved blood glucose control and

triglyceride levels (140). Resveratrol (8 mg/kg/d administered via osmotic pump) also ameliorated hepatic steatosis and lowered NLRP3 inflammasome levels (140). Misawa et al. (141) investigated resveratrol's inhibitory mechanism on the NLRP3 inflammasome. Acetylated α-tubulin facilitates the movement of mitochondria to the ER, which leads to the interaction between ASC (from the mitochondria) and the NLRP3 (on the ER), which activates the assembly of the NLRP3 inflammasome (141). Acetylated α-tubulin was decreased with 5 μM resveratrol treatment in mouse macrophages, consequently decreasing NLRP3 inflammasome activation (141).

EGCG is a polyphenol found in green tea. In lupus prone, New Zealand black/white (NZB/W) F1 mice, 120 mg/kg body wt EGCG inhibited ROS production and NF-κB activation in the kidney (142). EGCG-treated mice demonstrated lower IL-1β and IL-18 levels, as well as NLRP3 activation in the liver (142). EGCG lowered monocyte and macrophage infiltration to the liver and ameliorated prophylactic consequences of lupus nephritis in mice (142). EGCG (10 μM) decreases NLRP3 inflammasome activation in endothelial cells through increased AMPK phosphorylation, and decreased TXNIP activity and ROS production (91). These studies provide further proof of the benefit of dietary polyphenols.

1.6 Conclusions

Chronic inflammation mediates the detrimental consequences of auto-inflammatory conditions and obesity-related conditions. The NLRP3 inflammasome is a protein complex that is present in many types of cells and is activated by an extensive range of factors. After up-regulation of pro-inflammatory genes, the assembly of the

inflammasome may involve such cellular processes as K^+ efflux, lysosomal degradation, ER stress, and ROS production. The NLRP3 inflammasome has clearly been shown to be active in inflammatory conditions and is integral to the pathophysiology of many conditions including metabolic dysfunction, T2DM, NASH, and renal disease. NLRP3 activation can lead to insulin resistance and the NLRP3 inflammasome links obesity with many of the negative consequences of excess weight. Decreasing NLRP3 inflammasome expression and activation thus has many potential therapeutic applications.

The NLRP3 inflammasome is modulated by a variety of dietary factors. SFAs lead to NLRP3 inflammasome-related inflammation. On the other hand, UFAs and omega-3 fatty acids decreased NLRP3 inflammasome activation. Polyphenols, i.e. quercetin, curcumin, resveratrol, and EGCG, also attenuate inflammation by inhibiting inflammasome activation. These studies provide further support to the USDA dietary guidelines, which recommend a diet low in saturated fat, and higher in MUFAs and PUFAs, with plentiful fruits and vegetables (143). The Dietary Guidelines highlight the Mediterranean diet as an example of a healthful diet, characterized by high intakes of MUFAs and PUFAs, low intake of SFAs, and higher portions of whole grains, fruits and vegetables, and polyphenols (143). There is still much to learn about the NLRP3 inflammasome, and it is a promising method of ameliorating inflammation.

CHAPTER 2. EXPERIMENTS AND RESULTS

2.1 Introduction

The NRLP3 inflammasome has been implicated in a plethora of inflammatory diseases. In particular, activation of the NLRP3 inflammasome appears to be linked to chronic low-grade inflammation during obesity, and the development of obesity-induced insulin resistance. Therefore, our lab has investigated potential mediators of NLRP3 inflammasome activation. Specifically, our lab has examined the role of tocotrienols, which are known to be anti-inflammatory. Macrophages or "big eaters," are key immune cells intimately linked with inflammation in obesity and adipose inflammation as well. Though the NLRP3 inflammasome acts in many different cell types, macrophages are key factors in the innate immune response to overweight and obesity as well as a HFD. The outline of the mechanisms by which tocotrienols act will follow, as well as the role that macrophages play in chronic low-grade inflammation. Also discussed is the potential of tocotrienols to inhibit NLRP3 inflammasome activation.

A. Tocotrienols

Vitamin E is divided into two classes—tocopherols with a saturated side chain, and tocotrienols, that contain 3 double bonds in the side chain (144). α-, γ-, δ-, and β-tocotrienols differ based on the functional group at the 5 or 7 position on the chromanol ring (144). Tocotrienols are mainly found in vegetable oils including palm and rice bran oil, as well as oats, rye, barley, and other cereal grains (145). Palm oil is a common

source for tocotrienol supplements (146). The vitamin E content in palm oil is 30% tocopherols and 70% tocotrienols (146).

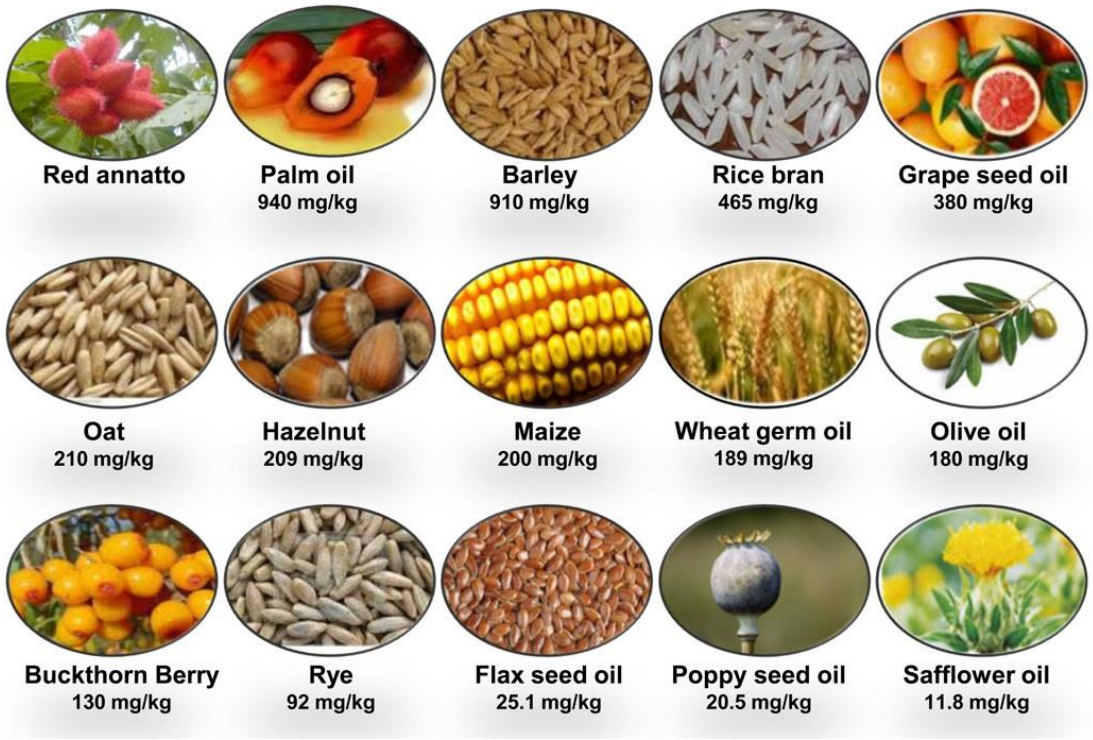
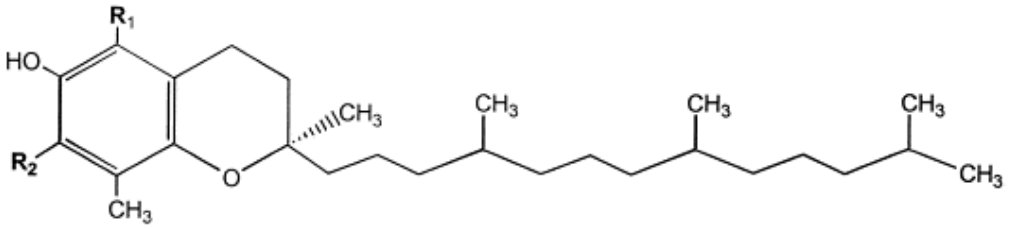
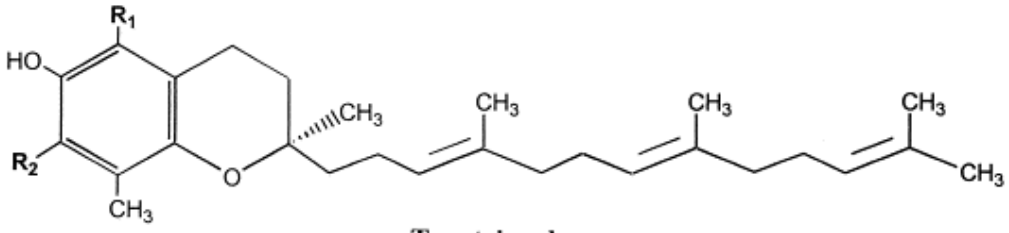


Figure 3: Tocotrienol content in various food sources (147)



Tocopherol



Tocotrienol

		R_1	R_2
α-Tocotrienol/ Tocopherol	**(aT3)**	CH_3	CH_3
β- Tocotrienol/Tocopherol		CH_3	H
γ- Tocotrienol/Tocopherol	**(gT3)**	H	CH_3
δ– Tocotrienol/Tocopherol	**(dT3)**	H	H

Figure 4. Tocotrienol structure (148)

a. Properties

Our lab has investigated tocotrienols for their anti-inflammatory effect. Tocotrienols are forms of vitamin E that exhibit antioxidant, anti-proliferative, anti-survival, pro-apoptotic, antiangiogenic, and anti-inflammatory activities (147). Tocotrienols are well-known antioxidants (149-159). Tocotrienols are more powerful antioxidants than tocopherols. Alpha-tocotrienol (aT3) is 40~60-fold more potent at mitigating lipid peroxidation in rat liver microsomal membranes and 6.5-fold more potent

at decreasing oxidative damage compared to alpha-tocopherol (aTP) (150). aT3 has greater antioxidant ability to decrease in vivo oxidative stress, possibly due to its increased effectiveness as an antioxidant in membranes (151). It is believed that the unsaturated side chain allows the tocotrienols to interact with lipid radicals more freely in the cell membrane (151). The well-documented antioxidant property of tocotrienols is induced through enzymes including superoxide dismutase (160,161). Vitamin E also induces NADPH: quinone oxidoreductase and glutathione peroxidase (162). The anti-oxidant properties of tocotrienols come in part from their ability to decrease TNF (163-165), IL-1β (166), IL-6 (167), and IL-8 levels (168). Tocotrienols ameliorate oxidative stress, chronic inflammation, and modulated apoptosis (165).

Tocotrienols are able to regulate cholesterol synthesis. Tocotrienols directly bind to 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and decrease cholesterol synthesis (147). In rats, supplementing tocotrienols (as the tocotrienol rich fraction (TRF) from palm oil) lowered LDL-cholesterol and total cholesterol due to the inhibitory effects on HMG-CoA reductase (169). Qureshi et al. (170) conducted an 8-week, double-blind crossover study with 25 human subjects with hypercholesterolemia. Tocotrienols (200 mg/day) lowered serum cholesterol by 31% within 4 weeks, and this decrease continued for 2 weeks, even after discontinuation of the supplement (170). Baliarsingh et al. (171) demonstrated that TRF supplement improved hyperlipidemia and atherogenesis in 19 patients with T2DM. TRF supplement for 60 days resulted in a reduction in serum total lipids, total cholesterol (TC), and LDL-C, although there was no improvement in glycemia in these subjects (171). However, not all studies found a cardioprotective effect of tocotrienol supplements (172). Some have proposed that the

differing outcomes could be due to the presence or absence of aTP in the supplements administered to the subjects (173). In chickens, aTP was shown to interfere the cholesterol-lowering effect of tocotrienols (174).

Another mechanism of tocotrienols work is inhibition of NF-κB activation. A combination of tocotrienols have been shown to inhibit NF-κB activation. Tocotrienols (α, β, γ) administration in pups with ethanol-induced impaired cognitive function decreased NF-κB p56 expression in the nuclear fraction (175). In mouse peritoneal macrophages, TRF was more powerful than aTP on inhibiting NF-κB expression, as well as decreasing LPS-induced nitric oxide, and TNF-α, IFN-γ, IL-1β, and IL-6 production (176). Similarly, TRF protected THP-1 cells from LPS-induced cell death through diminishing NF-κB expression alongside a decrease in TNF-α, IL-4, and IL-8 production (177). This inhibitory effect in immune cells was found when Theriault et al. (178) demonstrated that aT3 inhibited NF-κB activation by 30% in monocytes. In lymphocytes, gamma tocotrienol (gT3) pretreatment inhibited NF-κB activation and the production of the pro-inflammatory cytokine IFN-γ (179). Tocotrienols appear to be more effective than tocopherols at inhibiting NF-κB priming. Kuhad et al. (165) demonstrated that tocotrienol administration in STZ-injected mice prevented ROS-induced NF-κB p65 subunit expression in the nuclear fraction of STZ-treated rat kidneys. In their study, tocotrienols were more effective than aTP at decreasing NF-κB expression and inhibiting caspase-3 (165).

This alteration in NF-κB activation contributes to the anti-cancer and anti-inflammatory properties of tocotrienols. Tocotrienols inhibiting NF-κB expression in two human breast cancer cell lines (180). Tocotrienols have also been studied for cancer

protection, and it has anti-cancer effects in many different tumor cell types (147). Tocotrienols are anti-proliferative in breast, colon, liver, lung, pancreas, prostate, skin, and stomach tumor cells (147). This antiproliferative effect of tocotrienols is in part due to its inhibition of MAPK and ERK (181) and JNK (182). Tocotrienols may be potent in reducing cancer due to its angiogenesis suppressive property (183,184).

Tocotrienols are also beneficial for mitigating adverse effects of diabetes. Siddiqui et al. (185) demonstrated that TRF supplementation in STZ-injected diabetic rats protected against diabetic nephropathy. TRF supplementation improved glycemia, lowered HbA1c, decreased oxidative stress, lowered lipid peroxidation, and improved serum triglycerides (185,186). A placebo-controlled, randomized double-blind study with 44 type 2 diabetic patients examined the effects of a 200 mg/d tocotrienol supplement combined with noninsulin hypoglycemic medications (187). These researchers found that tocotrienols improve fasting blood glucose, through there was no improvement in HOMA-IR (187). Oral tocotrienol supplements in STZ-induced diabetic rats improved renal function, prevented lipid peroxidation and lowered TNF-α levels (165). The researchers proposed that these improvements were partly due to the action of tocotrienols of NF-κB signaling (165). Another study by Fang et al. (188) found that tocotrienol supplements reduced blood glucose levels in animal and patient models, potentially by acting on peroxisome proliferator-activated receptor (PPAR) modulators.

Tocotrienols prevented nicotine-induced bone loss (189) and improve normal bone structure and calcification (190,191). The beneficial effect of tocotrienol on bone structure may be due to its antioxidant property (192). Deng et al. (193) demonstrated that gT3 supplementation in C57BL/6 female mice treated and prevented osteoporosis,

and the gT3 accumulated in adipose, cardiac, and bone tissue. Tocotrienols are gastroprotective and lower free radical damage, and stress-induced elevated gastric acidity levels (194). Tocotrienols display a neuroprotective effect. This could be due to a few potential mechanisms—the antioxidant abilities of tocotrienols may decrease oxidative stress in the brain; tocotrienols may lower hypercholesterolemia, a known risk factor of neurodegenerative disease; or tocotrienols may affect neuron cell death (173). Moreover, the tocotrienol levels needed to achieve this neuroprotective effect may be achievable through the diet (173). It is evident that tocotrienols have widespread benefits in the body.

b. Digestion, Absorption, and Supplements

Tocotrienols are absorbed and transported in the body similarly to other fat-soluble vitamins. Tocotrienols are absorbed by Caco2 cells (colorectal adenocarcinoma cells) more readily than tocopherols due to the saturation of the side chains (195). Caco2 cells can be used as a cell model for lipoprotein metabolism (196). Oral administration of delta tocotrienol (dT3) (100 mg/kg body wt) in female athymic nude mice led to a peak plasma concentration of 57±5 μmol/l, which occurred 2 h after administration (197). α-tocopherol transfer protein (ATTP), α-tocopherol associated protein (TAP), P-glycoprotein (P-gly) and human serum albumin (HSA) may provide docking complex for uptake of tocotrienols and tocopherols into chylomicrons (198). Tocotrienols are cleared from the liver and plasma after 24 h (197). Tocotrienols are distributed in the body via chylomicrons alongside triglycerides, and are absent from the plasma after

chylomicrons are cleared (199). Carboxyethyl-hydroxychroman is the aT3 and gT3 metabolite that is excreted in human urine (200).

Oral tocotrienol supplementation in humans increases plasma tocotrienols to micromolar concentrations (201,202). The plasma concentrations of a-, g-, and dT3, reached 4 h after a high fat meal supplemented with 77 mg aT3, 96 mg dT3, 3 mg gT3 was 2.94 ± 0.68 μM, 3.16 ± 0.96 μM, and 1.41 ± 0.39 μM, respectively (202). Uchida et al. (203) demonstrated that tocotrienol is deposited in the adipose tissue and these stores remain in the adipose without degradation for 4 weeks after 6 weeks of supplementation. Male Sprague Dawley (SD) rats on a diet of 6.4 mg vitamin E/100g wt for 3 weeks were given non-, low-T3 (0.8 mg tocotrienol/kg body wt/d), and high-tocotrienol (3.2 mg tocotrienol/kg body wt/d) diets (204). The gT3 was significantly higher in the adipose tissue, and was increased from 1.1 to 10.2 nmol/g adipose tissue (204). Patel et al. (205) found that tocotrienol oral supplements significantly increased tissue levels of tocotrienol in the skin blood, brain, adipose, cardiac muscle, and liver in human subjects over time. The levels of aT3 found in the brain in this study were found to be neuroprotective (205).

Absorption of tocotrienols from intramuscular and intraperitoneal injections is negligible, but oral supplementation is more effective (206). aT3 bioavailability is ~28%; gT3 and dT3 bioavailability is ~9% (206). A tocotrienol intake of less than 0.20% of total dietary intake represents a safe amount in rats (207). Nakamura et al. (208) found the no-observed-adverse-effect level (NOAEL) of tocotrienols to be 0.019% in the diet, or 120 mg/kg body wt/d in male rats, and 130 mg/kg body wt/d in female rats. 300 mg/kg/d of dT3 or gT3 supplementation in male and female CD2F1 did not cause any adverse effects. Palm TRF are certified by the US FDA as a Generally Recognized as

Safe (GRAS; GRN307, October 2009) nutrients, and tocotrienols are not considered a drug that has potential side effects. However, there is not currently a recommended daily allowance (RDA) or tolerable upper limit (TUL) specifically for tocotrienols. Self-emulsifying formulas of tocotrienols have been tested in human subjects and were found to improve oral bioavailability through improving lipolysis (209). Tocotrienol supplements would therefore be safe in the human diets.

c. Delta Tocotrienol

dT3 is unique among other tocotrienols due to its high availability in the annatto plant. dT3 represents 90% of the tocotrienols found in the annatto plant, from which it is easily purified (210). Despite its high bioavailability in the annatto, dT3 has not been extensively studied in connection to inflammation and T2DM. As with the other tocotrienols, dT3 has anti-cancer and cardio-protective benefits.

One well-characterized action of dT3 is an inhibitory action on the NF-κB, which leads to its anti-cancer and anti-inflammatory actions. dT3 exhibited a growth-inhibitory effect and decreased NF-κB p65 in ED40515 cells (211). dT3 inhibited cell growth and migration, increased apoptosis, and decreased NF-κB DNA binding ability in cancer cells (212). In a pancreatic cancer model, dT3 was found to inhibit both pERK and NF-κB, which improved median survival (213). Husain et al. (214) also demonstrated dT3's ability to reduce tumor growth in human pancreatic cancer cells through inhibition of NF-κB activity and expression. STZ-injected diabetic rats supplemented with tocotrienols for 10 weeks exhibited behavioral and biochemical improvements related to diabetes (164).

The researchers demonstrated that these improvements were due to a decrease in NF-κB p65 expression in the nuclear fraction of the rats' brains (164).

dT3 has been shown to have anti-cancer effects in pancreatic cancer (213-215), breast adenocarcinoma (216), non-small cell lung cancer (214,217,218), lymphoblastic leukemia (219), and melanoma (220). Compared to aTP, dT3 demonstrated a greater ability to suppress angiogenesis and inhibit proliferation in human umbilical vein endothelial cells (HUVECs) (221). In mice, an annatto-T3 supplement reduced breast cancer tumor mass, and lung metastases size through growth arrest and apoptosis, ROS production and gene expression associated with cancer development (210). dT3 effectively inhibited human colorectal adenocarcinoma growth through apoptosis and phosphorylation of protein kinase B (Akt) (222). dT3 arrested cell growth in human bladder cancer cells (223). dT3 treatment was found to lower ROS production and suppress melanogenesis through MAPK/ERK signaling (224). dT3 also protects human cells from radiation-induced damage through down-regulation of microRNA-30c and IL-1β expression (225). Li et al. (225) demonstrated that this protective effect was through inhibited NF-κB signaling. Singh et al. (226) investigated the dT3 radio-protective ability in CD2F1 mice, and found that it was due to dT3's ability to induce granulocyte colony-stimulating factor (G-CSF). Further, dT3 protects against radiation-induced gastrointestinal damage in CD2F1 mice (227). These researchers also found that dT3 reduced translocation of gut bacteria, protected intestinal cells, and decrease levels of IL-1β and IL-6 (227).

Rats fed a diet high in simple carbohydrates displayed obesity, insulin resistance, and hypertension (228). However, dT3 supplementation improved cardiovascular

markers, hypertension, plasma lipids, blood glucose, and insulin signaling (228). Though these rats displayed low to no levels of tocotrienol in the plasma, tocotrienols were deposited in adipose, liver, and heart tissue (228). Supplementation with gT3 and dT3 in hypercholesterolemic mice reduced cholesterol and triglyceride synthesis and improved serum triglycerides (229). Abdul-Majeed et al. (230) demonstrated that dT3 in SD rats is an effective treatment to prevent post-menopausal osteoporosis. dT3 has been shown to be effective in ameliorating collagen-induced rheumatoid arthritis (231). Though dT3 has mostly been studied for its anticancer effects, it has potential beneficial effects in diabetes and cardiovascular diseases.

d. Tocotrienols and the NLRP3 Inflammasome

gT3 has been shown to mitigate inflammation associated with a HFD. Supplementing mice fed a HFD with gT3 ameliorated adipose inflammation, plasma levels of inflammatory cytokines, and IL-6 and IL-1β gene expression (232). This was due to NF-κB inhibition. Supplementing gT3 also improved insulin signaling, which demonstrates that gT3, through NFκB inhibition, decreases HFD-induced adipose inflammation and improves metabolic dysfunction (232). Our lab has demonstrated that gT3 is anti-inflammatory in adipose tissue and is able to alter the macrophage profile in adipose. gT3 supplementation in C57BL/6J mice fed a HFD decreased epididymal fat pad size (232). Insulin resistance was improved in the gT3-supplemented mice as well (232). Additionally, macrophage infiltration into the adipose, macrophage pro-inflammatory polarization, and adipocyte inflammation were lowered with gT3 supplementation (232). This led to a subsequent study on the mechanisms leading to this

decrease in inflammation. gT3 inhibited NLRP3 inflammasome activation in LPS- and nigericin- or palmitate-activated NLRP3 inflammasome in iJ774 macrophages (233). In db/db mice, gT3 supplementation improved glycemia and β-cell mass (233). gT3-supplemented db/db mice displayed lower levels of NLRP3 inflammasome activation in peritoneal macrophages (233). Thus, it was concluded that gT3 is able to inhibit the NLRP3 inflammasome.

B. The Role of the Macrophage and the Immune System in Inflammation

The macrophage is commonly used as a model for NLRP3 inflammasome activation. It is particularly relevant when studying the innate immune response and ensuing inflammation during metabolic dysfunction and obesity. Macrophages are central to adipose inflammation and thus represent an ideal target to ameliorate chronic low-grade inflammation. Danger signals during overweight and obesity activate the innate immune system and lead to sterile inflammation. Diet-induced obesity leads to lower levels of $CD4^+$ helper and regulatory T cells and infiltration of epididymal adipose tissue by $CD8^+$ effector T cells, which recruit macrophages to the adipose (234). Infiltration of T cells and macrophages in visceral adipose tissue leads to chronic inflammation and contributes to the development of obesity-induced insulin resistance (235).

The immune system is involved with the pathogenesis of obesity induced diseases. In mice, a HFD induced infiltration of macrophages and dendritic cells, even before weight increased and insulin resistance declined, demonstrating that macrophage infiltration to the adipose is an early step in the development of obesity-induced insulin

resistance (130). During obesity, macrophages are recruited to the adipose tissue and instigate inflammatory pathways that lead to low-grade inflammation in the adipose (236). This is due to the production of MCP-1 by adipose in response to a HFD and leads to the recruitment of macrophages to the adipose (11). A HFD in NLRP3 knockout mice led to lower MCP-1 and consequently lower macrophage infiltration into adipose tissue (4). Wild-type mice on the HFD had higher levels of macrophage infiltration, which leads to adipocyte inflammation and HFD-induced insulin resistance (4). Macrophage cells comprise 10% of adipose tissue in lean mice and humans, which increases dramatically to 40% in obese humans and 50% in the adipose of leptin-deficient mice (236).

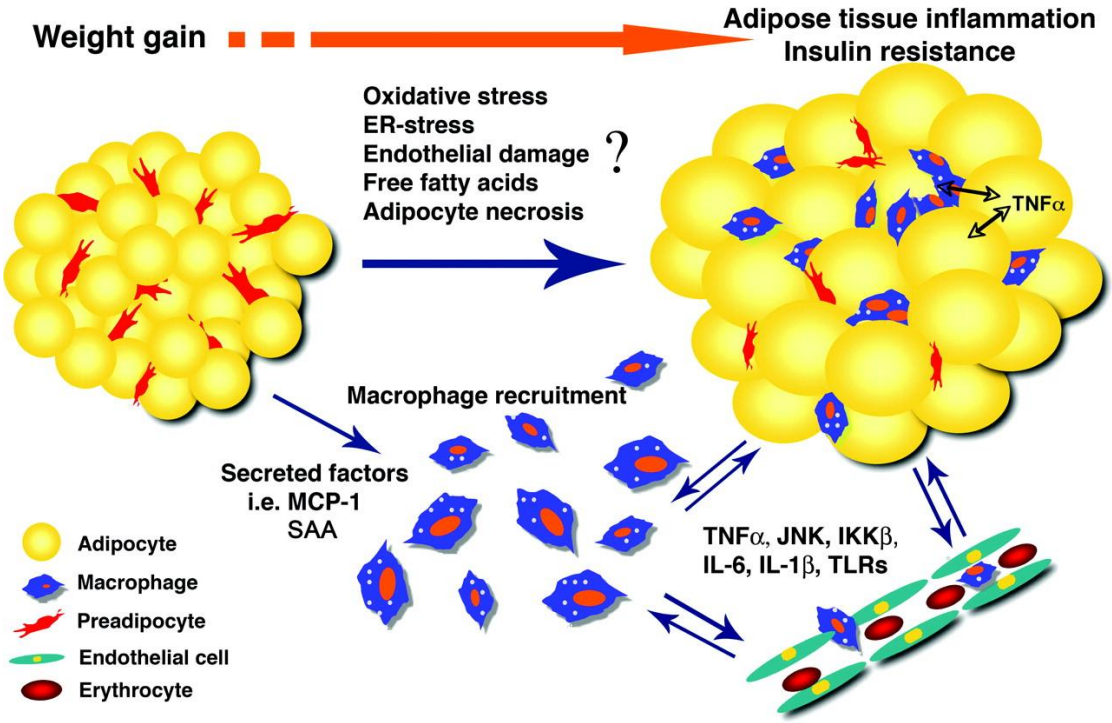


Figure 5. The role of macrophages in inflammation. Weight gain during obesity leads cellular stress, which results in the recruitment of macrophages to adipose tissue. These macrophages secrete pro-inflammatory cytokines and inflammatory factors, resulting in decreased insulin sensitivity. (237)

A HFD leading to elevated fatty acids activates macrophage NLRP3 inflammasome, which was associated with impaired insulin signaling (121). Obesity is marked by the polarization of anti-inflammatory M2 macrophages to the pro-inflammatory M1 macrophage recruitment to adipose tissue (11). These M1 macrophages are connected to the development of insulin resistance and produce pro-inflammatory cytokines (11). Diet-induced obesity alters levels of $CD4^+$ helper and regulatory T cells in adipose, and these cells influence glucose homeostasis, adipose hypertrophy, and obesity (238). Macrophages are key players in the development of atherosclerosis. NLRP3 inflammasome activation facilitated the dramatic uptake of lipids into bone marrow macrophage lysosomes (16). ATP-stimulated NLRP3 inflammasome activation resulted in high levels of cholesterol accumulation in the lysosome (16). NLRP3 inflammasome activation also altered trafficking of intracellular lipids, which caused a buildup of sphingolipids in the lysosome (16). The inflammasome increased macrophage migration in an IL-1β- and IL-18-independent manner. The researchers hypothesized that inflammasome activation enable macrophages to invade the artery wall, where the formation of foam cells leads to atherosclerosis (16). Activation of the NLRP3 inflammasome in macrophages plays a role in atherogenesis and adipose inflammation.

C. Gaps in Knowledge

dT3, aT3, and gT3 are structurally similar and may possess the same anti-inflammasome properties. dT3 is readily isolated from the annatto plant, and may represent a potential therapeutic compound for ameliorating diabetic complications. The

well-delineated anti-oxidant, anti-inflammatory, and pro-apoptotic properties of tocotrienols make them an ideal candidate for inhibiting the NLRP3 inflammasome. The NLRP3 inflammasome has been implicated in diabetes (17,113,121). Tocotrienol supplementation improves blood glucose levels and other detrimental effects of T2DM (165,188). Indeed, our lab has found that gT3 inhibits the NLRP3 inflammasome (233). gT3 supplementation in a mouse model improved diabetes outcomes (233).

Tocotrienols have been established as having anti-inflammatory and anti-oxidant properties. Tocotrienols lower pro-inflammatory cytokines, such as TNF (163,165), IL-1β (166), IL-6 (167), and IL-8 levels (168). Tocotrienols induce superoxide dismutase (160,161) and NADPH:quinone oxidoreductase and glutathione peroxidase (162), which enables tocotrienols to alleviate oxidative stress. Though exact NLRP3 inflammasome activation mechanisms are yet to be fully elucidated, ROS production has been identified as a common factor in NLRP3 inflammasome activators (24,27). ROS inhibtors have been shown repeatedly to inhibit NLRP3 inflammasome activation (25,59,72-74,87). ROS production causes the protein TXNIP to bind with NLRP3 inflammasome, and assist inflammasome activation (55). Consequently, our lab investigated the role of tocotrienols in ROS production in inflammasome inhibition in macrophages. Tocotrienols have been demonstrated to inhibit NF-κB (164,165,175-180). dT3 has been studied for its ability to lower NF-κB priming, particularly in cancer models (211-213). Our lab therefore determined whether tocotrienols played a role in inhibiting NF-κB activation in macrophages stimulated with LPS as a potential mechanism for inflammasome activation inhibition. Macrophages are key immune cell players in the development of obesity-induced inflammation (121). Macrophages are recruited to

adipocytes during metabolic dysfunction (236). They present NLRP3 inflammasome activity and thus are an ideal model to study for reducing chronic low-grade inflammation (121).

In this research project, the potential of tocotrienols to decrease NLRP3 inflammasome activation in macrophages was studied. NF-κB activation and ROS production were investigated as potential mechanisms for NLRP3 inflammasome inhibition.

2.2. Central Hypothesis, Purpose, and Specific Aims

A. Purpose of the Study

The purpose of the study is to investigate the role of tocotrienols (dT3, aT3, gT3) on NLRP3 inflammasome activation in macrophages and determine potential mechanisms of action behind the relationship between tocotrienols and inflammasome, focusing on dT3.

B. Central Hypothesis

Tocotrienols (dT3, aT3, gT3) inhibit NLRP3 inflammasome activation and IL-1β secretion in macrophages by decreasing NF-κB activation and ROS production.

C. Specific Aims

a. Specific Aim 1

Establish the role of dT3 on NLRP3 inflammasome activation and IL-1β production in macrophages and compare the effect of dT3 in NLRP3 inflammasome activation to other tocotrienol and tocopherol forms.

The hypothesis for this aim is that dT3 will dose-dependently decrease NLRP3 activation IL-1β in macrophages. We hypothesize that dT3 and gT3, but not aT3 will inhibit NLRP3 inflammasome activation and IL-1β secretion in macrophages.

b. Specific Aim 2

Investigate whether tocotrienols (dT3, aT3, gT3) inhibit the NLRP3 inflammasome through inhibition of NF-κB signaling and ROS production

We hypothesize that tocotrienols (dT3, aT3, gT3) inhibit NF-κB activation and lower ROS production in macrophages, resulting in the inhibition in NLRP3 inflammasome activation.

2.3. Materials, Methods, and Experimental Design

A. Materials

Preparation of tocotrienols

American River Nutrition (Hadley, MA, USA) provided dT3. Carotech (Edison, NJ,USA) provided gT3; 90% purity and aT3; 90% purity. The concentration was determined by measuring observance at 292 nm and 298 nm using Synergy H1 multi-mode reader (BioTek), and comparing it to a standard. dT3 was stored as 3.3 mM stock, gT3 and aT3 were stored as 1 mM stock solution prepared in dimethyl sulfoxide (DMSO), in 100 μL aliquots, at -20^{0}C. The stock was diluted to 1-10 μM at the time of treatment to iJ774 macrophages and raw 264.7 macrophages.

Nigericin

Nigericin (a K^+/H^+ ionophore) derived from *Streptomyces hygroscopicus* was purchased from Sigma-aldrich. 6.5 mM Nigericin stock was prepared in ethanol and stored at 20°C.

Lipopolysaccharide

Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 was stored at 20°C and diluted with completed media immediately before treatment to iJ774 macrophages.

J774 cells

J774 (iJ774) macrophages were a generous gift from Dr. Hornung. iJ774 iGLuc macrophages express IL-β-Gaussia Luciferase fusion construct (iGLuc). Gaussia luciferase (GLuc) activity was detected using the BioLux GLuc assay kit (NEB Inc.) (according to manufacturer's instructions) (239).

Raw 264.7 Macrophage Cells

Raw 264.7 macrophage cells are from Abselon murine leukemia virus-induced tumor, from an adult male BALB/c mouse. Raw 264.7 macrophages were purchased from ATCC (TIB-71).

B. Methods

Cell Culture

All cells were cultured either in completed media or Opti-MEM. Cultured media consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1.5 mM L-glutamine, sodium pyruvate, 10% fetal bovine serum (Gibco), and 5% penicillin/streptomycin. Cells were maintained at 5% CO2 at 37 °C.

Determination of Tocotrienol Concentration

Tocotrienol stock was diluted with dimethyl sulfoxide (DMSO). Stock solution was diluted 25x, 50x, and 100x, then observance was measured at OD292 nm and OD298 nm using Synergy H1 multi-mode reader (BioTek). Ethanol was used as the blank. The extinction coefficient ε (the OD value of 1M) was used to calculate concentration per (163).

OD292 ε aT3=3870 $M^{-1}cm^{-1}$

OD292 ε dT3=3300 $M^{-1}cm^{-1}$

OD298 ε gT3=4230 $M^{-1}cm^{-1}$

Results were averaged for the final stock concentration.

Reporter Assay for Inflammasome

IL-β-Gaussia Luciferase fusion construct (iGLuc) activity was determined with the BioLux GLuc Assay kit (NEB Inc.) and read using a Synergy H1 multi-mode reader (BioTek). 50 µL of BioLux GLuc substrate solution (NEB Inc.) was added to 20 µL of each sample, and luminescence was measured using Synergy H1 multi-mode reader (BioTek), according to manufacturer's instructions.

Measurement of IL-1β Production

Levels of IL-1β in the medium was determined with commercial ELISA kits from R&D Systems. Manufacturer's protocol was followed.

Western blot

Immunoblotting was performed to measure protein expression in samples. To measure caspase-1 and iGLuc proteins in collected media, 24 µL of media were loaded into the gel. For total cell extracts, cells were first with Hank's Balanced Salt Solution (HBSS) and cell extract collected using ice-cold Radioimmune Precipitation Assay (RIPA) buffer

(89% RIPA, 10% phosphatase inhibitor, 1% proteinase inhibitor). The isolate was sonicated, centrifuged, and protein concentration determined with bicinchoninic acid assay (BSA assay). 15 μg of each sample was loaded in a 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gels. Wet transfer was used to transfer the gel to a polyvinylidene difluoride (PVDF) membrane. 5% non-fat milk in tris-buffered saline with tween-20 (TBST) was used as washing buffer. Membrane was next washed with TBST and incubated with primary antibody overnight. The next day, the membrane was washed with TBST, incubated with secondary antibody (anti-rabbit, -mouse, or –goat) for 1 h, then washed with TBST. Membranes were developed using chemiluminesence using ECL solution (PerkinElmer) with a FluorChem E system (Proteinsimple). Polyclonal or rabbit monoclonal antibodies targeting p-JNK (Thr183/Tyr185), p-ERK(Thr202/Tyr204), t-ERK, and inhibitor of kappa B (IκBα) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody targeting iGLuc was purchased from New England BioLabs (Ipswich, MA, USA). Antibody targeting caspase-1 was purchased from Adipogen (San Diego, CA, USA). Band intensity was determined using ImageJ software.

Real time qPCR

qPCR will be performed to analyze gene expression. Cells were homogenized in Trizol reagent (Invitrogen). mRNA was isolated using chloroform and isopropanol. DNase (Mediatech) was used to remove any genomic DNA contamination. mRNA was reprecipitated, and quantified. Bio-Rad iSCRIPT cDNA synthesis kit was used to generate 100 μL cDNA, stored at -20°C. SYBR green and forward and reverse primers were added to each sample cDNA. QuantStudio 6 Flex System was utilized for qPCR.

Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. Gene expression was normalized to HPRT.

Table 1: Forward and Reverse Primers

Gene	Forward	Reverse
HPRT	GCTATAAATTCTTTGCTGACCTGCTG	AATTACTTTTATGTCCCCTGTTGACTGG
IL-18	GACAGCCTGTGTTCGAGGAT	TGGATCCATTTCCTCAAAGG
IL-1β	GTCACAAGAAACCATGGCACAT	GCCCATCAGAGGCAAGGA
NLRP3	ATGCTGCTTCGACATCTCCT	AACCAATGCGAGATCCTGAC
TNFα	GGCTGCCCCGACTACGT	ACTTTCTCCTGGTATGAGATAGCAAAT

Measurement of Reactive Oxygen Species Generation

Cells were washed with HBSS with Ca^{2+} and Mg (1.5 mM calcium, 1 mM magnesium). MitoSOX reagent was dissolved in DMSO, then diluted in HBSS with Ca^{2+} and Mg for a 5 µM solution. Cells were incubated with MitoSOX for 10 minutes protected from light. It is a fluorogenic molecular probe that targets mitochondria and reacts with superoxide to emit fluorescence.

Statistical Analysis

Statistical analysis included student's t-test or one-way ANOVA with Tukey's multiple comparison tests. All statistical analyses were conducted by GraphPad Prism 6 (Version 6.02). Statistical significance was defined as $p<0.05$. In figures, *$p<0.05$, **$p<0.01$, ***$p<0.001$

C. Experimental design

a. Specific Aim 1

Study 1: Investigate the effectiveness of varying doses of dT3 on inhibiting NLRP3 inflammasome activation and IL-1β secretion. The hypothesis is that dT3 will dose-dependently decrease NLRP3 inflammasome activation and IL-1β secretion in macrophages

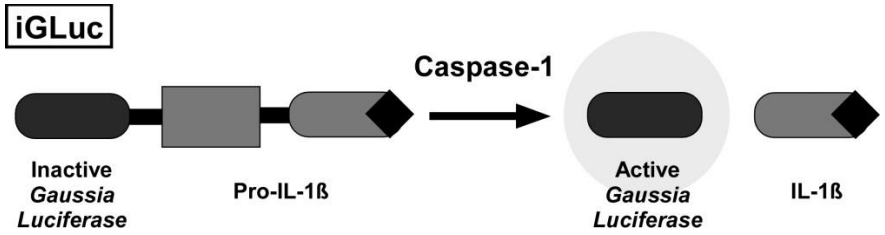


Figure 6. Gaussia luciferase construct in iJ774 macrophage reporter cell line

Study 1 Detailed Procedure:

iJ774 macrophages were seeded to 24-well plate and cultured with Opti-MEM media (n=4). Cells were pre-treated with 0.5, 1, 2.5 and 5 μM dT3 or DMSO (control). After 48 h, media was changed and 0.5, 1, 2.5 and 5 μM dT3 or DMSO was added. The NLRP3 inflammasome was stimulated with 100 ng/mL LPS for 1 h. NLRP3 inflammasome was activated with 6.5 μM Nigericin for 1 h. Media was collected and stored in -80°C freezer.

Pro-caspase 1 cleavage to caspase-1 cleavage was analyzed using Gaussia luminescence. 50 μL of Gaussia luciferase assay was added to 20 μL media and the luminescence was measured using the luminometer function of the Synergy H1 multi-mode reader (BioTek). IL-1β levels in the media were determined using commercial ELISA kits from R&D System, following the manufacturer's protocol. Results reported in pg/mL.

Western blot using media collected was used to verify the presence of iGLuc and to measure caspase-1 in the media.

Study 2: Compare the effectiveness in various tocotrienols (dT3, aT3, gT3) on NLRP3 inflammasome activation and IL-1β secretion in macrophages. The hypothesis for this study is that dT3 and gT3 will effectively inhibit NLRP3 inflammasome and IL-1β secretion, and aT3 will not inhibit NLRP3 inflammasome and IL-1β secretion in macrophages

Study 2 Detailed Procedure:

iJ774 macrophages were seeded to 24-well plate and cultured with Opti-MEM media (n=4). Cells were pre-treated with 1 μM dT3, aT3, or gT3 or DMSO (control). After 48 h, media was changed and tocotrienols were added. The NLRP3 inflammasome was stimulated with 100 ng/mL LPS for 1 h. NLRP3 inflammasome was activated with 6.5 μM Nigericin for 1 h. Media was collected and stored in -80°C freezer.

Pro-caspase 1 cleavage to caspase-1 cleavage was analyzed using Gaussia luminescence. 50 μL of Gaussia luciferase assay was added to 20 μL media and the luminescence was measured using the luminometer function of the Synergy H1 multi-mode reader (BioTek). IL-1β levels in the media were determined using commercial ELISA kits from R&D System, following the manufacturer's protocol. Results reported in pg/mL.

Western blot using media collected measured the presence of iGLuc and to measure caspase-1 in the media.

The hypothesis is that dT3 will block NF-κB activation

b. Specific Aim 2:

Study 1: Determine the effect of various doses of dT3 on NF-κB activation in raw 264.7 macrophages. The hypothesis is that dT3 would dose-dependently decrease NF-κB activation stimulated by LPS in macrophages

Study 1 Detailed Procedure:

Raw 264.7 macrophages were cultured in a 6-well plate in opti-MEM (n=3). Cells were pre-treated with 1 or 2.5 μM dT3 or DMSO for 48 h. NF-κB activation was stimulated with LPS (100 ng/mL) for 3 h. qPCR as detailed earlier was used to measure gene expression of NLRP3, TNF-α, IL-1β, and IL-18. Reference gene was HPRT.

Study 2: Determine the effect of different tocotrienols (dT3, aT3, gT3) on NF-κB activation in raw 264.7 macrophages. The hypothesis is that tocotrienols would inhibit NF-κB activation stimulated by LPS in macrophages

Study 2 Detailed Procedure:

Raw 264.7 macrophages were cultured in a 6-well plate in opti-MEM (n=3). Cells were pre-treated with 1 μM dT3, aT3, or gT3 or DMSO for 48 h. NF-κB activation was stimulated with LPS (100 ng/mL) for 3 h. qPCR as detailed earlier was used to measure gene expression of NLRP3, TNF-a, IL-1β, and IL-18. Reference gene was HPRT.

Raw 264.7 macrophages were cultured in a 6-well plate in opti-MEM (n=2). Cells were pre-treated with 1 μM dT3, aT3, or gT3 or DMSO for 48 h. NF-κB activation was stimulated with LPS (100 ng/mL) for 30 minutes. Total cell extract was isolated as detailed earlier for Western Blot. Samples were analyzed for pERK, t-ERK, p-JNK, p-p38, and p- IκBα.

Study 3: Investigate the ability of tocotrienols to diminish ROS production in LPS/Nigericin stimulated raw 234.7 macrophages. The hypothesis is that dT3, aT3, and gT3 would lower ROS production in raw 234.7 macrophages activated by LPS/Nigericin

Live Cell Imaging:

Raw 264.7 macrophages were seeded 2.5x10^5 cells in 12 well plate, and pre-treated with DMSO or 1 µM tocotrienol (dT3, aT3, gT3) for 48 h (n=2). Cells were treated with LPS (100 ng/mL) for 1 h. Media was removed and cells washed with HBSS with Ca^{2+} (1.5 mM) and Mg^{2+}(1 mM). 5 µM MitoSOX solution (MitoSOX reagent dissolved in DMSO and diluted in HBSS with $Ca^{2+}$and Mg^{2+}) was added to cells and incubated protected from light for 10 minutes. MitoSOX solution was removed, and 100ng/mL LPS plus 6.5 µM Nigericin with 1µM tocotrienols was added. Cells were incubated away from light for 10 minutes. Cells were washed with HBSS with $Ca^{2+}$and Mg^{2+}. Nuclei were counter-stained in blue using DAPI, and pictures were taken using the EVOS FL cell imaging microscope (ThermoFisher, Waltham, MA, USA) on 40X magnification.

Fluorescence Quantification:

Raw 264.7 macrophages were seeded 30,000 cells/well in a 96 well plate and pre-treated with DMSO or 1 µM tocotrienol (dT3, aT3, gT3) for 48 h (n=6). Cells were treated with LPS (100 ng/mL) for 1 h. Media was removed and cells washed with HBSS+ with $Ca^{2+}$and Mg^{2+}. 5 µM MitoSOX solution (MitoSOX reagent dissolved in DMSO and diluted in HBSS with $Ca^{2+}$and Mg^{2+}) was added to cells and incubated protected from light for 10 minutes. MitoSOX solution was removed, cell are stimulated with media containing 100ng/mL of LPS with 6.5 µM Nigericin and 1µM tocotrienol. Cells were

incubated away from light for 10 minutes. Cells were washed with HBSS with $Ca^{2+}$and Mg^{2+}. Fluorescence excitation and emission was measured at 510 nm and 580 nm by Synergy H1 multi-mode reader (BioTek).

2.4. Experimental Design and Results

A. dT3 inhibits NLRP3 inflammasome activation in iJ774 macrophages.

To determine the impact of dT3 on inflammasome activation, NLRP3 inflammasome reporter assay was conducted, iJ774 macrophages that stably overexpress an inflammasome reporter (239) were pretreated with increasing doses of dT3 (0-5 μM) or vehicle (DMSO) for 48 h. Cells were primed with LPS for 1 h and the inflammasome was stimulated by adding nigericin. Gaussia luciferase activity reflected caspase-1 mediated pro- IL-1β cleavage and gauged NRLP3 inflammasome activation. Pretreatment with 0.5-5 μM dT3 significantly decreased NLRP3 inflammasome activation (Fig. 7A). Consistent with decreased inflammasome activation, dT3 pre-treatment strikingly lowered IL-1β secretion to medium in iJ774 macrophages (Fig. 7B). These data clearly revealed that 1 μM of dT3 effectively attenuated IL-1β secretion and NLRP3 inflammasome activation.

To further confirm these results, we measured protein levels of caspase-1 and iGLuc in the medium. iGLuc protein is secreted from macrophages upon caspase-1 cleavage of pro-IL-1β. Treatment with dT3 higher than 1 μM abolished iGLuc and caspase-1 secretion to the medium (Fig. 7C), suggesting almost complete blockage of inflammasome activation. Band intensity quantification reflected this drastic decrease (Fig. 7D-E). These results indicated that dT3 pretreatment suppresses LPS/nigericin-

stimulated NLRP3 inflammasome activation, IL-1β production, and caspase-1 activity in iJ774 macrophages.

B. Tocotrienols inhibit NLRP3 inflammasome activation in iJ774 macrophages.

In a previous study, we demonstrated that gT3 was an effective suppressor of NLRP3 inflammasome activation in primary bone marrow derived macrophages as well as in iJ774 macrophages (239). We next asked whether other tocotrienol forms, i.e., aT3, gT3, and dT3 that share the unsaturated side chain, inhibited NLRP3 inflammasome activation. Since the majority of published studies used mixed tocotrienols, it would be useful to delineate the effectiveness of individual tocotrienol in inhibiting inflammasome activation.

To address this, we pre-treated iJ774 macrophages with 1 μM dT3, α-tocotrienol (aT3), or γ-tocotrienol (gT3) or DMSO (vehicle) for 48 h. Since 1 μM of tocotrienol is achievable in serum after oral intake of tocotrienol in humans (201,202), we chose 1 μM of individual tocotrienol as the pretreatment conditions. iJ774 macrophages were first primed with LPS for 1 h and stimulated with nigericin for additional 1 h. Gaussia luciferase activity revealed that all tocotrienols, i.e., dT3, aT3, gT3, were similarly effective in inhibiting NLRP3 inflammasome activation (Fig. 8A). Unexpectedly, only dT3 and gT3, but not aT3, significantly diminished IL-1β secretion to the media as measured by ELISA (Fig. 8B). aT3 tended to lower IL-1β secretion to the medium, but the difference did not reach statistical significance (Fig. 8B).

To confirm the inhibitory effects of inflammasome by tocotrienols, secretion of caspase-1 and iGLuc protein to the media was measured. We found that gT3 inhibited

caspase-1 and iGLuc secretion the most potently (Fig. 8C). Band intensity confirmed that gT3 decreased caspase-1 and iGLuc protein levels the most potently. Taken together, these results demonstrate that dT3, aT3, and gT3 pretreatment in macrophages inhibit LPS/nigericin- stimulated NLRP3 inflammasome activation, but shows a difference in potency as follows: gT3>dT3>aT3.

C. dT3 decreases NF-κB activation in raw macrophages.

After observing that dT3 inhibits NLRP3 inflammasome in macrophages, we investigated the underlying mechanisms. The priming step of NLRP3 inflammasome involves NF-κB priming, which acts to provide transcripts of pro-inflammatory genes (49). In our experimental setting, NF-kB activation occurs through TLR4 signaling, a pattern recognition receptor by LPS. Literature suggests that dT3 inhibits NF-κB activation in cancer models (211-213).

Thus, we first investigated the effect of dT3 on down-stream target genes of NF-κB activation including NLRP3, TNF-α, IL-1β, and IL-18 mRNA gene levels. Raw 264.7 macrophages were pre-treated with vehicle control (DMSO), 1 or 2.5 μM of dT3 for 48 h. Cells were treated with LPS for 3 h to initiate NF-κB activation. qPCR data showed that mRNA gene expression of *Nlrp3*, *Tnf-α*, and *Il-1b* was significantly decreased by 1 μM and 2.5 μM pretreatment of dT3 (Fig. 9A-C). IL-18 mRNA expression was significantly lowered by pretreatment of 2.5 μM dT3 (Fig. 9D). We concluded that 1 μM and 2.5 μM dT3 pre-treatment decreases pro-inflammatory NF-κB target gene expression when macrophages are primed with LPS.

D. Tocotrienols decrease NF-κB activation in raw macrophages.

There is a body of evidence that aT3, gT3, and dT3 inhibit NF-κB activation in cancerous cells (164,165,175-180). We therefore asked whether this property extended to macrophages. Next, we wanted to determine the extent to which NF-κB inhibition by aT3, gT3 and dT3 contribute to the suppression of NLRP3 inflammasome in macrophages. We pretreated raw 264.7 macrophages with 1 μM dT3, aT3, or gT3 or DMSO (vehicle) for 48 h, then stimulated with LPS for 3 h to induce NF-κB activation. Pretreatment with dT3, aT3, gT3 significantly decreased *Nlrp3*, *Tnf-α*, and *Il-1b* mRNA gene expression, measured by qPCR (Fig. 10 A-D). *Il-18* gene levels were significantly decreased by dT3 and gT3.

To further confirm NF-κB inhibition by tocotrienol for down-regulation of NLRP3 inflammasome activation, we measured NF-κB signaling protein levels by Western blot analysis using total cell lysate (Fig. 10E). IκBα is the cytosolic protein that sequestrates NF-κB from nuclear translocation and thereby inhibits NF-κB activation. IκBα was degraded when NF-κB priming was activated through LPS stimulation. Pretreatment with dT3 and gT3 protected from LPS-induced NF-κB activation. Band intensity demonstrated that dT3 and gT3 significantly increased IκBα protein levels, whereas aT3 tended to increase IκBα, though this was not statistically significant (Fig. 10F). P-ERK was increased with LPS activation, whereas cells treated with dT3, aT3, and gT3 significantly down-regulated expression levels of P-ERK. The decrease in P-ERK levels due to pretreatment with dT3, aT3, and gT3 was statistically significant (Fig. 10G). JNK is inactivated with NF-κB activation (240). P-JNK levels were significantly higher in the aT3, and gT3 pretreated groups, and lower in response to LPS stimulation

(Fig. 10H). dT3 did not significantly lower P-JNK levels (Fig. 10H). dT3 appears to lower NF-κB activation independent of P-JNK. Taken together, these results confirmed that NF-κB activation is down-regulated in macrophages pre-treated with dT3, aT3, and gT3 and stimulated with LPS. gT3 appears to be consistently the most effective at decreasing NF-κB-related gene expression and protein levels.

E. Tocotrienols decrease ROS production in raw macrophages.

Finally, we tested if tocotrienols inhibit NLRP3 inflammasome activation by lowering ROS production. ROS is a common event that occurs in NLRP3 activation by a variety of stimulants (27). Cassel et al. and Ding et al. (25,72) both reported that blockage of ROS production inhibited NLRP3 inflammasome activation. Tocotrienols are powerful antioxidants—more powerful than tocopherols—which provides a rationale that tocotrienols attenuate NLRP3 inflammasome activation via inhibition of ROS production in macrophages (149-159,241-244).

We pre-treated raw 264.7 macrophages with 1 μM dT3, aT3, or gT3 or DMSO (vehicle) for 48 h, primed with LPS for 1 h, and activated the inflammasome for 10 minutes with nigericin. MitoSOX was used to measure ROS production. Nuclei were counter-stained in blue with DAPI (4',6-diamidino-2-phenylindole). Inflammasome activation by LPS/nigericin treatment increased ROS production while pretreatment with dT3, aT3, and gT3 significantly attenuated MitoSOX red fluorescence suggesting that tocotrienols lowers ROS production (Fig. 11A). To quantify this decrease in ROS, raw 264.7 macrophages were pretreated with 1 μM dT3, aT3, or gT3 or DMSO (vehicle) for 48 h, primed with LPS for 1 h, and activated the inflammasome for 10 minutes with

nigericin (Fig. 11B). MitoSOX was used to measure ROS levels. Quantification of red fluorescence revealed a significant decrease in ROS production when cells were pretreated with aT3 and gT3 (Fig. 11B). dT3 pretreatment showed a trend to decrease in ROS production, but it was not significant (Fig. 11B). Taken together, these results indicate that pretreatment with aT3, gT3, but a lesser extent with dT3, decrease ROS production during LPS/nigericin-mediated inflammasome activation in macrophages.

2.5. Discussion

A. Discussion

The NLRP3 inflammasome is a multi-protein complex that is assembled in response to cellular stressors (13). NLRP3 inflammasome activation results in the secretion of the inflammatory cytokines IL-1β and IL-18 (75). It perpetuates chronic low-grade inflammation and is implicated in the development of insulin resistance (121), atherosclerosis (6), and liver steatosis (34) among many other inflammatory conditions. Tocotrienols are recognized as being anti-inflammatory and anti-oxidants, and dT3 is an easily isolated form of tocotrienol (147). We therefore primarily aimed to determine whether dT3 inhibited NLRP3 inflammasome activation, and compare the inhibitory effect of dT3 on the NLRP3 inflammasome to other tocotrienol forms, i.e., aT3 and gT3. Secondly, we investigated potential mechanisms of NLRP3 inflammasome attenuation. In our model of tocotrienols and inflammasome activation, a priming signal (e.g. LPS, FFA) activates NF-κB signaling and increases pro-inflammatory gene expression (Fig. 8). The second signal consisting of DAMPs and PAMPs initiate the assembly and activation of the NLRP3 inflammasome and involves increased ROS production (Fig. 8).

Activation of the NLRP3 inflammasome leads to production of pro-inflammatory cytokines. However, dT3, aT3, and gT3 inhibit the NF-κB priming signal and decrease ROS production in LPS/nigericin primed macrophages (Fig. 8).

We chose macrophages as the model for NLRP3 inflammasome activation due to the involvement of the immune system, and in particular macrophages, in the development of obesity-related diseases. Therefore, the inhibition of inflammasome activation in macrophages is potentially metabolically beneficial. A high fat diet can lead to NLRP3 inflammasome activation in macrophages (121). In addition, overweight and obesity leads to macrophage infiltration in the adipose and adipocyte inflammation (236). This suggests that high circulating levels of free fatty acids during a high fat diet and the overweight and obese states send a danger signal to macrophages. The NLRP3 inflammasome then becomes activated in the macrophages and they begin to infiltrate the adipose tissue where they perpetuate the inflammatory signal (130). Adipose inflammation then leads to peripheral insulin resistance, which worsens as the chronic inflammation continues (130). Therefore, we investigated the therapeutic potential of tocotrienols in alleviating the unwarranted activation of the NLRP3 inflammasome.

We showed that pretreatment with 0.5-5 μM dT3 inhibits LPS/nigericin-induced NLRP3 inflammasome activation (Fig. 7). This led to a decrease in IL-1β production and caspase-1 levels in macrophages (Fig. 7B-C). This inflammasome-inhibitory effect extends to other tocotrienols. dT3, aT3, and gT3 all inhibit NLRP3 inflammasome activation and IL-1β production in macrophages stimulated with LPS/nigericin (Fig. 8). We hypothesized that aT3 would not inhibit NLRP3 inflammasome activation based on earlier data that aT3 not to be effective at inhibiting NF-κB activation. Therefore, we

were surprised that aT3 inhibited NLRP3 inflammasome activation and IL-1β secretion. To our knowledge, this is the first study demonstrating that aT3 and dT3 also have an inhibitory effect on NLRP3 inflammasome activation. Besides mediating the development of insulin resistance, the NLRP3 inflammasome continues the inflammatory cascade in many auto-inflammatory conditions. Therefore, these tocotrienols may have ameliorate aberrant inflammation in these conditions. Because of the central role that macrophages in infiltrating adipose tissue and mediating adipocyte inflammation, inhibiting inflammasome activation in macrophages with tocotrienols may be beneficial in the chronic, low-grade inflammation present during overweight and obesity. We next investigated potential mechanisms of NLRP3 inflammasome inhibition. NF-κB activation is involved in the priming of the NLRP3 inflammasome (49). ROS production has been implicated in many NLRP3 inflammasome activators (27).

NF-κB inhibition by tocotrienols has been studied in various cancer cell models, but their role in inhibiting NF-κB priming in macrophages has not been clearly established. TLR signaling leads to NF-κB activation, which up-regulates genes including NLRP3, TNF-α, IL-1β, and IL-18 and NF-κB, which ultimately increases activation of NLRP3 inflammasomes (3,26,49). For example, LPS can signal through TLR4 (7). Our results indicate that tocotrienols decrease NF-κB activation in macrophages, leading to NLRP3 inflammasome inhibition (Fig. 10). 1-2.5 μM dT3 diminished down-stream gene targets of NLRP3 inflammasome activation including *Nlrp3, Tnf-α, Il-1b,* and *Il-18* (Fig. 9). dT3, aT3, and gT3 all inhibited up-regulation of the pro-inflammatory genes of *Nlrp3, Tnf-α, Il-1b,* and *Il-18* (Fig. 10A-D). Tocotrienol pretreatment maintained cytosolic levels of IκBα in LPS-primed macrophages. From our

results, it appears that gT3 is the most potent inhibitor of NF-κB activation in macrophages. This difference between tocotrienol forms may be due to the difference in the side chains.

After determining the role of NF-κB priming, we asked whether tocotrienols inhibited ROS production during the assembly stage of NLRP3 inflammasome activation. ROS is one of many NLRP3 inflammasome activators (27). Mitochondrial damage and ROS production is related to Ca^{2+} flux (94), and K^{+} efflux leads to damage to the mitochondria (28). ROS play a role in the deubiquitination of basally expressed NLRP3 inflammasome and subsequent activation (96). ER stress can lead to increased ROS production (67). In macrophages, tocotrienols appear to be anti-oxidants by lowering ROS levels. We found that dT3, aT3, gT3 inhibit ROS production in LPS/nigericin-stimulated macrophages (Fig. 11). Live cell imaging demonstrated a decrease in ROS production by dT3, aT3, and gT3 in macrophages stimulated with LPS/nigericin (Fig. 11). Fluorescence quantification showed that only aT3 and gT3 significantly lowered ROS production. Though dT3 diminished ROS production, it was not statistically significant. gT3 appears to be the most effective at decreasing NF-κB activation and ROS production. dT3's inhibitory mechanism may rely more heavily on NF-κB inhibition, whereas aT3 is more potent at lowering ROS production. These results demonstrate that tocotrienols can alleviate oxidative stress due to stimulation with LPS and nigericin.

The result of NF-κB priming inhibition and diminishment of ROS production in macrophages was ultimately a decrease in IL-1β production. The IL-1 cytokine family, and in particular IL-1β, is heavily studied due to its activity in auto-inflammatory

diseases (245). White adipose tissue is a main site of IL-1Ra, the IL-1β receptor, and the level of IL-1Ra is up-regulated during obesity and inflammation (103). This supports the hypothesis that adipose tissue is a key organ in mediating inflammation through IL-1β signaling during obesity (103). IL-1β is implicated in the development of insulin resistance and the pathophysiology of T2DM (246). High levels of IL-1β in pancreatic β-cells promotes apoptosis and cell death, which may lead to insulin resistance and the development of T2DM (247). Blocking IL-1β cytokine signaling has long been proposed as a mechanism for improving insulin sensitivity. In KK-Ay mice, blocking IL-1β improved circulating levels of free fatty acids and triglycerides, lowered β-cell apoptosis, and ultimately improved glycemia and insulin signaling (248). Decrease of IL-1β production in macrophages by tocotrienols could thus improve insulin resistance and mitigate adipocyte inflammation. Addition of tocotrienols may also protect against β-cell death. Tocotrienols could ameliorate the detrimental inflammatory response to a diet high in saturated fats.

Chronic inflammation is implicated in the pathophysiology of many obesity-related diseases. The NLRP3 inflammasome is at the crux of this metabolic inflammation, and is involved in many auto-inflammatory diseases. Decreasing aberrant inflammation may ameliorate these conditions. Lower levels of secreted IL-1β are potentially beneficial for many chronic diseases, such as T2DM. To our knowledge, this is the first study delineating the differential effects of the tocotrienols dT3, aT3, and gT3 on NLRP3 inflammasome activation in macrophages. Here, we have shown that tocotrienols effectively inhibit NLRP3 inflammasome activation and IL-1β production through attenuating NF-κB priming and ROS production suggesting that inclusion of

tocotrienol in the diet may constitute a practical approach to attenuate and/or prevent inflammation-mediated metabolic disorders, such as obesity and diabetes.

B. Limitations and Future Studies

There are several limitations to this study. This was an *in vitro* study, so the results obtained from dT3 and aT3 treatment may not exactly translate in an *in vivo* model. Furthermore, though measurement of IκBα protein degradation levels correlate with NF-κB activation, determination of NF-κB nuclear translocation more directly measures this priming step. We were not able to correct MitoSOX fluorescence data for mitochondrial membrane potential, and this reagent only measures superoxide formation. It would be useful to measure other types of ROS in the cells.

A possible future study would be to measure ROS production through DCFDA, which captures a wider variety of ROS produced in the cell. Other future studies could investigate the role of tocotrienols in ameliorating ER stress. Tocotrienols may also decrease K^+ efflux, or modulating Ca^{2+} which may inhibit NLRP3 inflammasome activation. NLRP3 inflammasome activation is modulated through microRNA (97,98), and this is another mechanism that could be investigated. Another possible study could examine the cross-talk between macrophages and adipose tissue, focusing on the effect on tissue inflammation and insulin signaling. This study could also investigate adipose browning, adipogenesis, and glucose tolerance.

Tocotrienols could be studied in many in vivo models of inflammatory conditions that involve the NLRP3 inflammasome. These include as arthritis, gout, and Alzheimer's disease. In vivo models could test whether gT3 or a combination of tocotrienols are more

effective at ameliorating inflammation. One study could examine whether a combination of tocotrienols or solely gT3 is more effective at ameliorating the adipose inflammation associated with a diet high in saturated fat. This research could also investigate whether there are metabolic benefits to the liver. Tocotrienols may affect liver inflammation and decrease NASH, since it is related to NLRP3 inflammasome activation. In addition, adipose inflammation decreased adipocyte browning, so a study could investigate whether tocotrienols may facilitate the browning by inhibiting inflammation. An *in vivo* study could test whether tocotrienol supplements improves endothelial dysfunction in a model of cardiovascular diseases. Potentially, human trials could investigate the efficacy of a tocotrienol supplement in attenuating NLRP3 inflammasome activation in adipose and the effect on insulin resistance and inflammation. Tocotrienols could also be studied in a human trial investigating the benefit of tocotrienols in conditions associated with the NLRP3 inflammasome including gout, arthritis, or Alzheimer's disease.

Figures

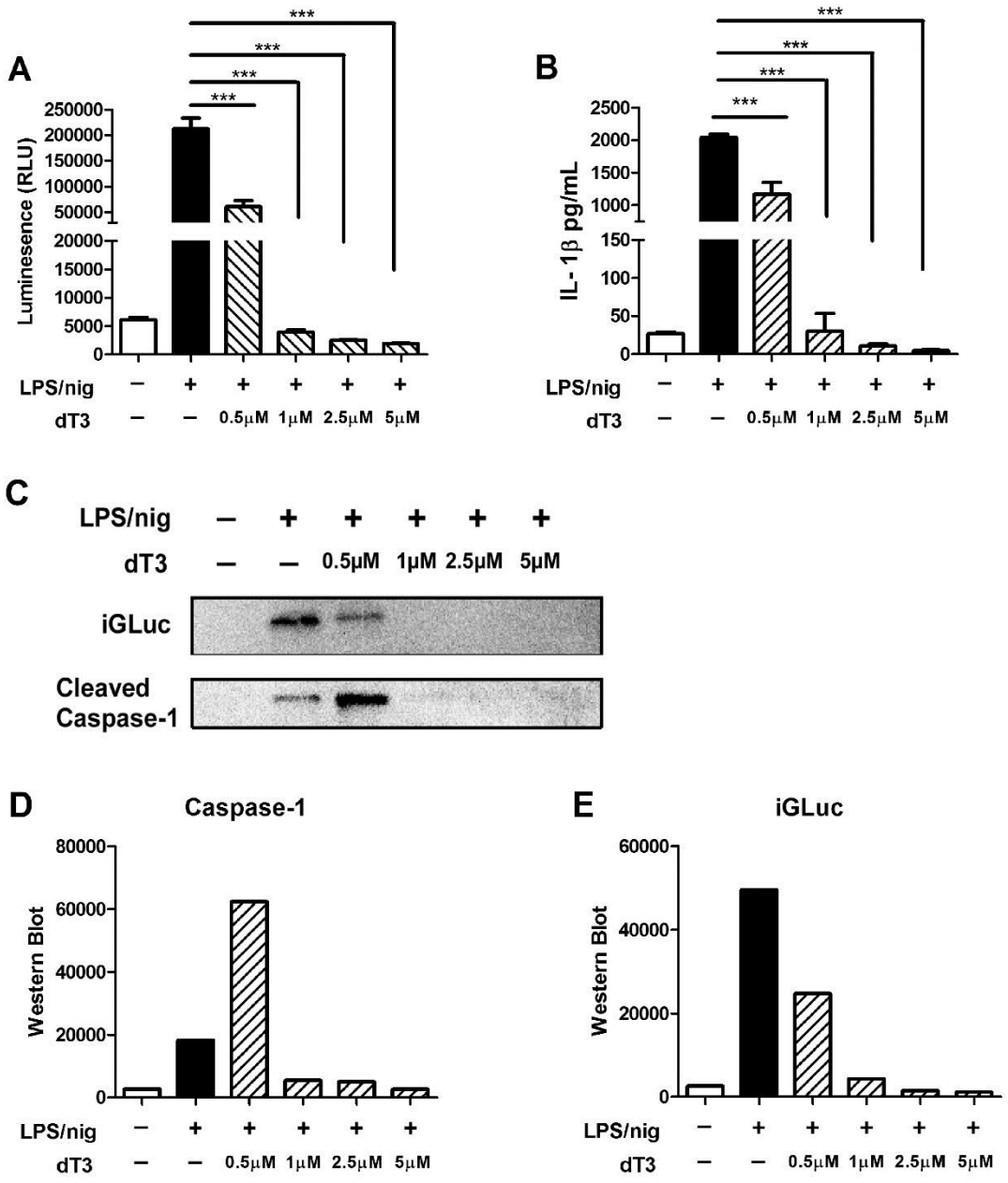


Figure 7. dT3 inhibits NLRP3 inflammasome activation in iJ774 macrophages. iJ774 macrophages were pre-treated with dT3 (0-5 μM) for 48 h, then primed with LPS (100 ng/mL) for 1h and stimulated with Nigericin (nig) (6.5 μM) for 1 h. A: Relative Gaussia luciferase activity measured by luminometer. B: IL-1β secretion in supernatant, quantified by ELISA. C: Supernatant protein levels of GLuc fusion protein and cleaved caspase-1 detected through Western blot. D-E. Band intensity of caspase-1 and iGLuc Western blot, quantified by ImageJ.

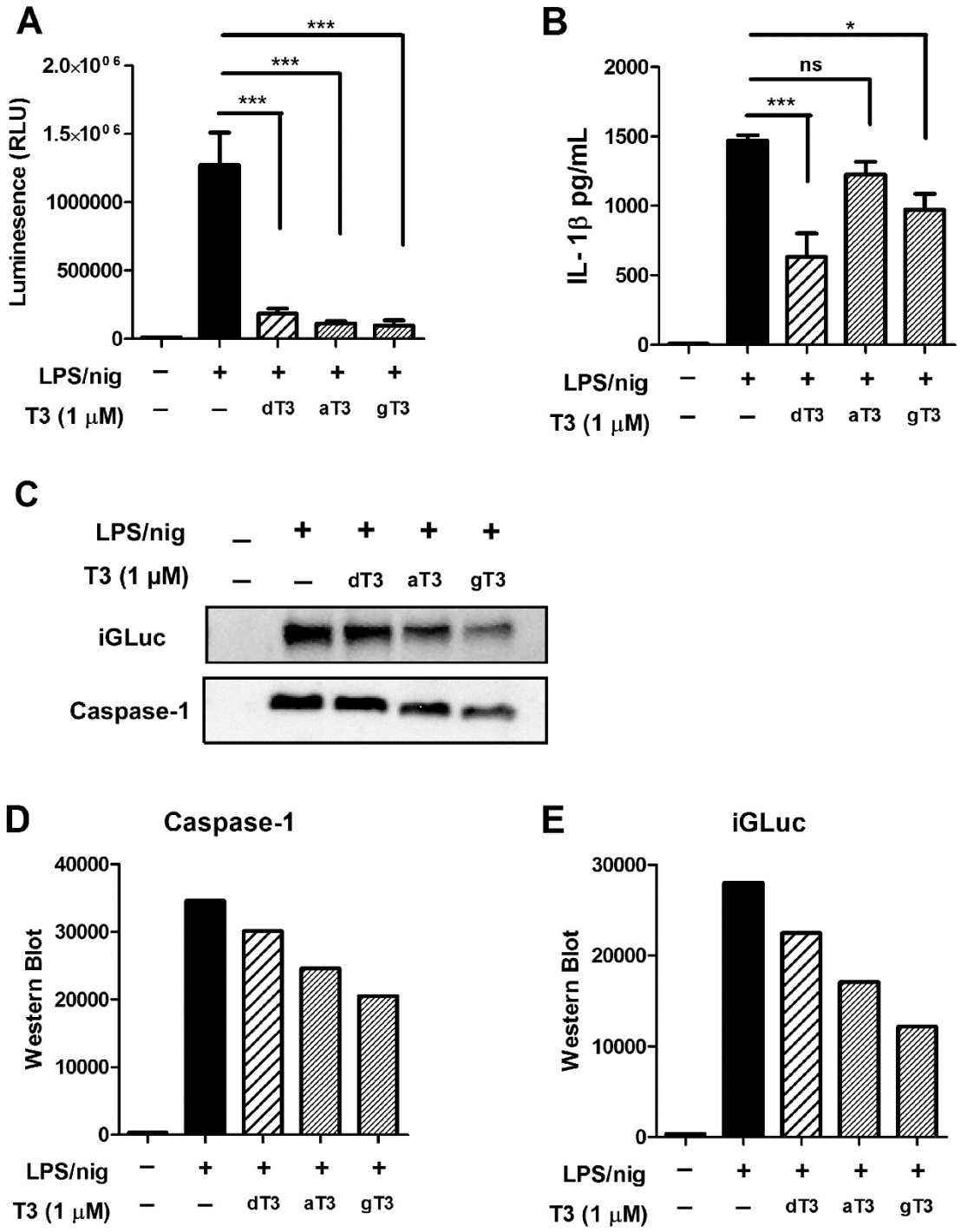


Figure 8. Tocotrienols inhibit NLRP3 inflammasome activation in in iJ774 macrophages. iJ774 macrophages were pre-treated with dT3, aT3, and gT3 (1 μM) for 48 h, then primed with LPS (100 ng/mL) for 1h and stimulated with Nigericin (nig) (6.5 μM) for 1 h. A: Relative Gaussia luciferase activity measured by luminometer. B: IL-1β secretion in supernatant, quantified by ELISA. C: Supernatant protein levels of GLuc fusion protein and cleaved caspase-1 detected through Western blot. D-E. Band intensity of caspase-1 and iGLuc Western blot, quantified by ImageJ.

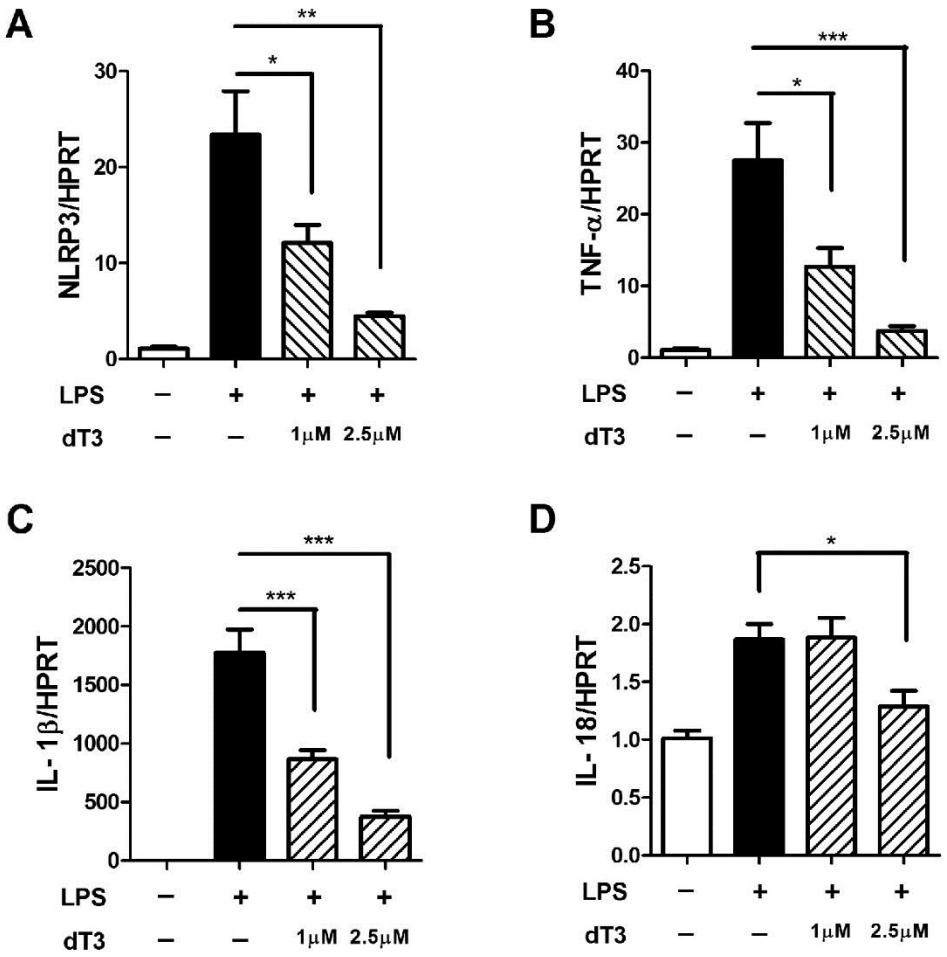
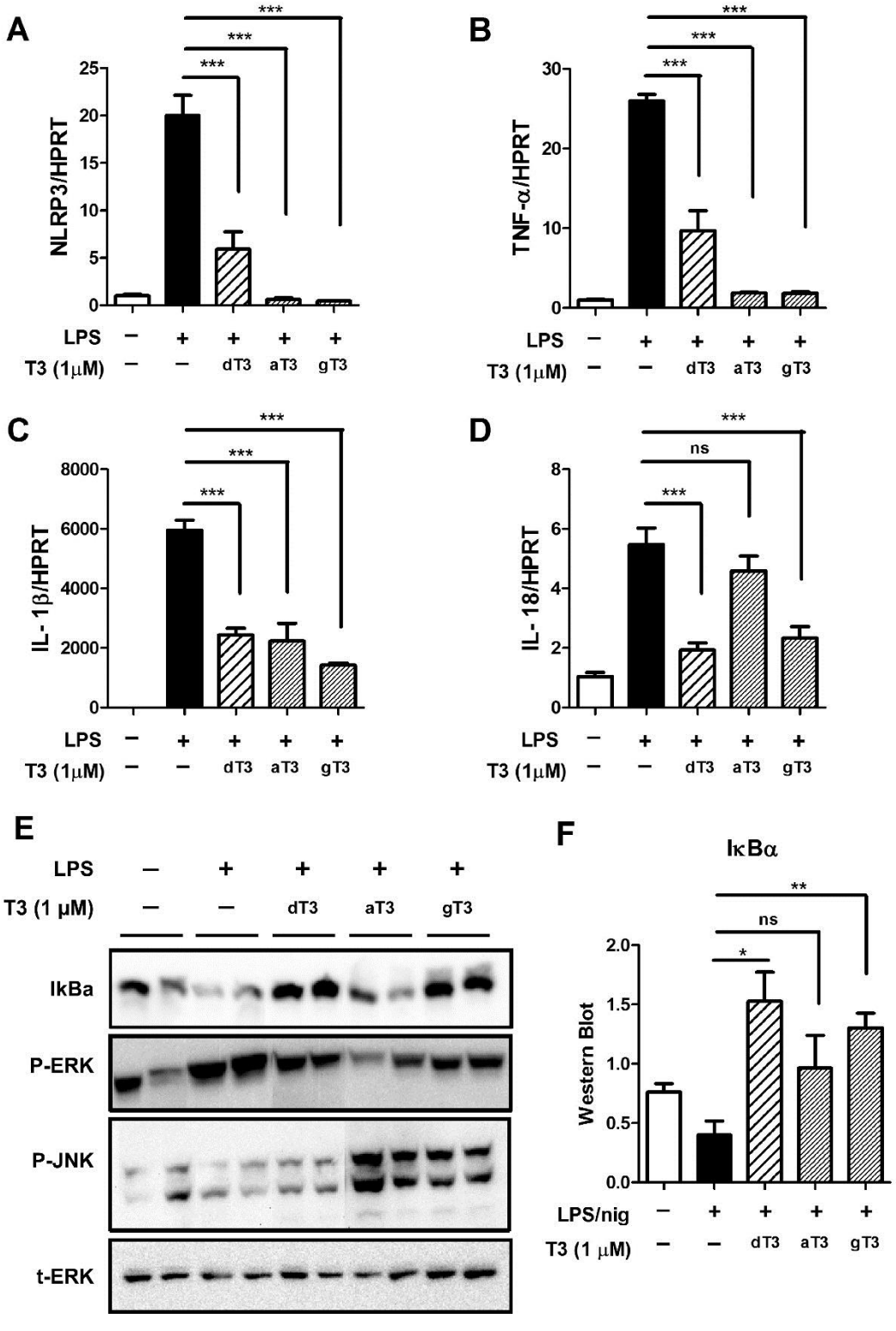


Figure 9. dT3 decrease NF-κB activation in manner in raw macrophages. Raw 264.7 macrophages were pre-treated with dT3 (0-5 μM) for 48 h, then primed with LPS (100 ng/mL) for 3h. A-D: Gene expression measured through qPCR


A
NLRP3/HPRT
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LPS – + + + +
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TNF-α/HPRT
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LPS – + + + +
T3 (1μM) – – dT3 aT3 gT3
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LPS – + + + +
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D
IL-18/HPRT
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P-JNK
t-ERK
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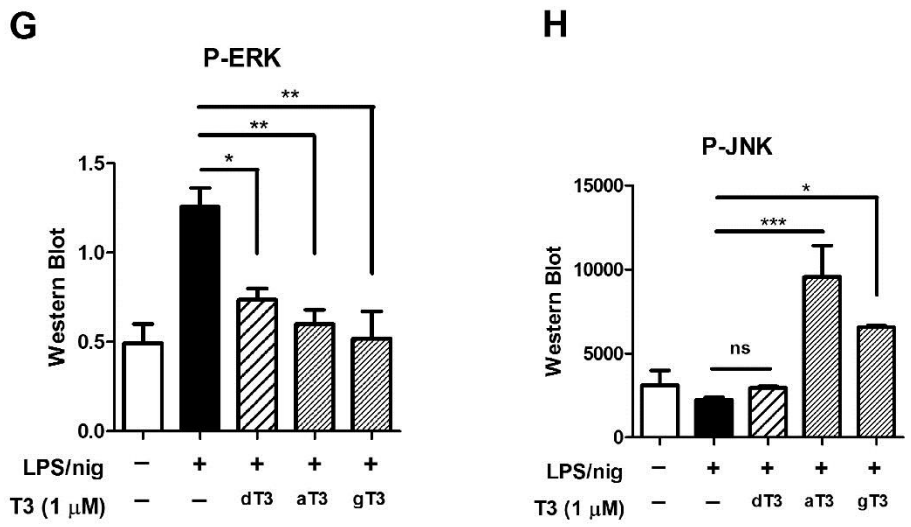
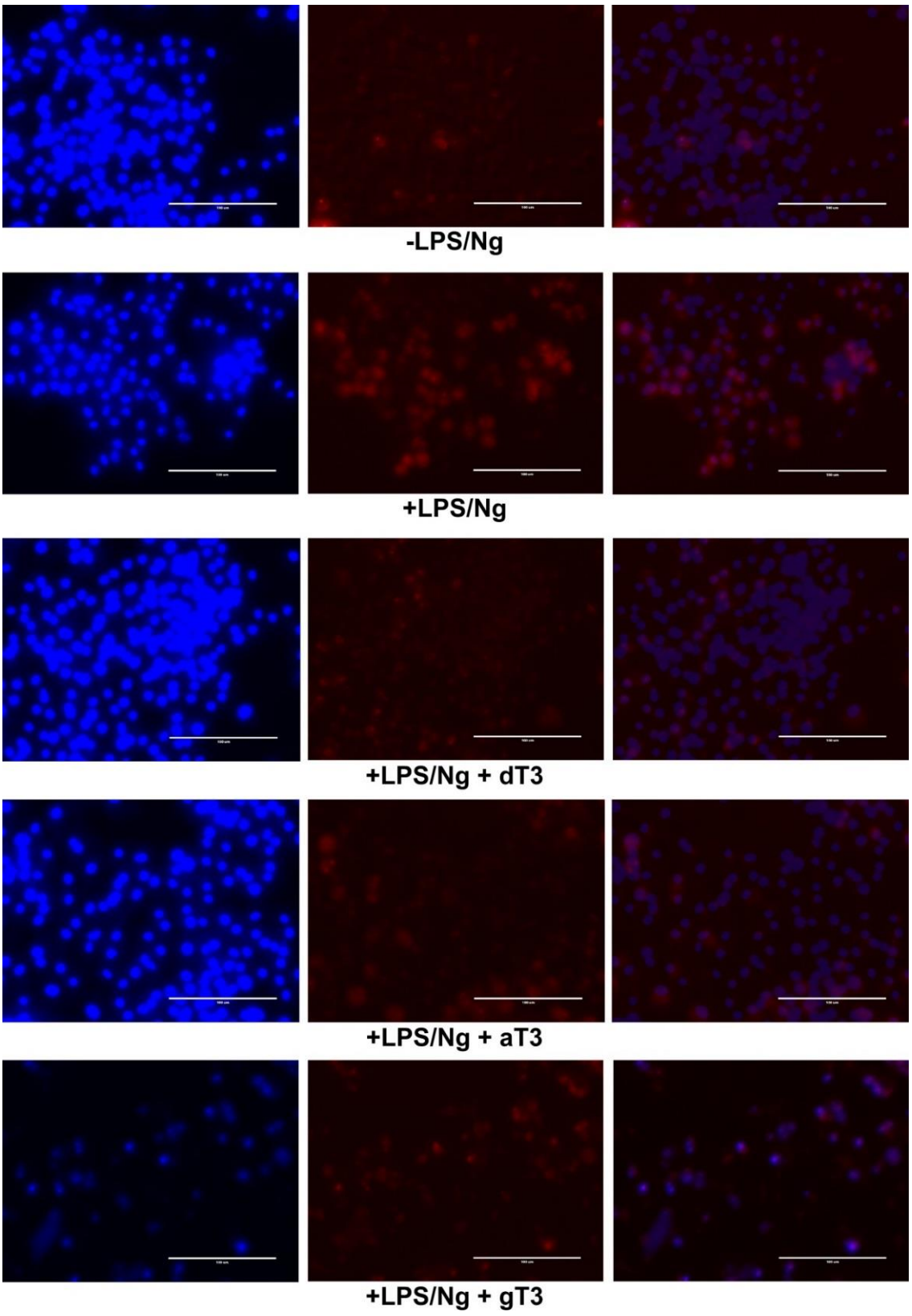


Figure 10. Tocotrienols decrease NF-κB activation in raw macrophages. Raw 264.7 macrophages were pre-treated with dT3, aT3, and gT3 (1 μM) for 48 h, then primed with LPS (100 ng/mL) for 3h. A-D: Gene expression measured through qPCR. E: Protein levels measured with Western Blot F-H. Band intensity of IκBα, P-ERK, and P-JNK Western blot, quantified by ImageJ.

A



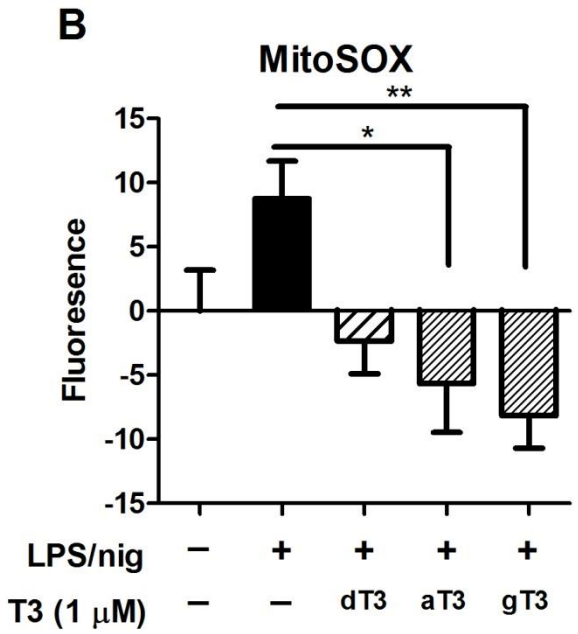


Figure 11. Tocotrienols decrease ROS production in raw macrophages. Raw 264.7 macrophages were pre-treated with dT3, aT3, and gT3 (1 μM) for 48 h, then primed with LPS (100 ng/mL) for 1 hour and activated with Nigericin (nig) (6.5 μM) for 20 minutes. Reactive oxygen species was detected using MitoSOX reagent. A: Nuclei were stained blue. Cell images were take using EVOS FL cell imaging microscope. B: Fluoresence

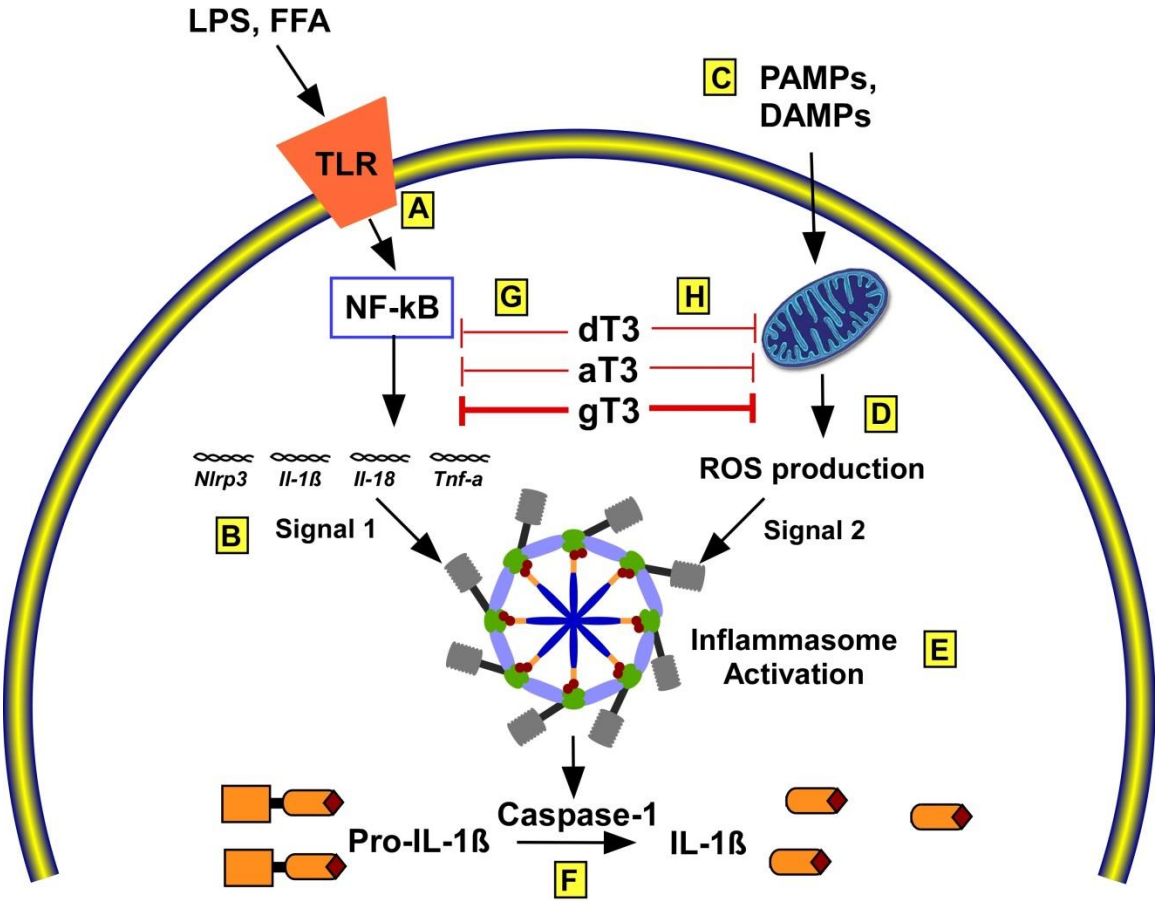


Figure 12. Proposed model that tocotrienols attenuate NLRP3 Inflammasome. An initial signal (e.g. LPS, FFA) signals through TLRs to induce NF-κB signaling (A) and increase pro-inflammatory gene expression (i.e. *Nlrp3, Il-1β, Il-18, Tnf-α*) (B). PAMPs or DAMPs constitute the second signal (C) and induce ROS production (D), which initiates inflammasome assembly and activation (E). This leads to caspase-1-mediated cleavage of IL-1β (F). Tocotrienols (i.e. dT3, aT3, gT3) inhibit NF-κB activation, reducing pro-inflammatory gene expression (G). As a secondary mechanism, tocotrienols inhibit ROS production, which attenuates the second activating signal for NLRP3 inflammasome assembly (H). The proposed potency for tocotrienols on NLRP3 inflammasome inhibition is gT3>dT3>aT3 (G,H). Inhibition of NLRP3 inflammasome activation results in reduced production of the pro-inflammatory cytokine IL-1β (F).

APPENDIX 1:

Abbreviations

AMPK—AMP-activated protein kinase
APAP—Acetaminophen
ARRB2—Arrestin, Beta 2
ASC—Apoptosis-associated speck-like protein containing CARD
aT3—Alpha-tocotrienol
ATF6—Activating transcription factor 6
ATP—Adenosine triphosphate
ATTP—α-tocopherol transfer protein
ATM—Adipose tissue macrophages
ATRvD1—Aspirin-triggered Resolvin D1
BMDM—Bone marrow derived macrophages
BSA— Bicinchoninic acid
cAMP—Cyclic adenosine monophosphate
CARD—Caspase activation and recruitment domain
C/EPB—CCAAT-enhancer-binding protein
CHOP—C/EBP homologous protein
CLA—Conjugated linoleic acid
COX—Cyclooxygenases
CPPD—Calcium pyrophosphate dehydrate
DAMP—Damage-associated molecular patterns
DAPI—4',6-diamidino-2-phenylindole
DHA—Docosahexaenoic acid
DMEM—Dulbecco's modified eagle medium
DMSO—Dimethyl sulfoxide
dT3—Delta-tocotrienol
EGCG—Epigallocatechin-3-gallate
EPA—Eicosapentaenoic acid
ER—Endoplasmic reticulum
ESRD—End-stage renal disease
FFA—Free fatty acids
FFAR4—Free fatty acid receptor 4
FSGS—Focal segmental glomerulosclerosis
G-CSF—Granulocyte colony-stimulating factor
GPR—G-protein coupled receptor
GRAS—Generally recognized as safe
gT3—Gamma-tocotrienol
HbA1c—Hemoglobin A1c
HBSS—Hank's balanced salt solution
HFD—High-fat diet
HK-2—Human kidney-2
HMGB1—High mobility group box 1
HMG-CoA—3-hydroxy-3-methylglutaryl-coenzyme A
HOMA-IR—Homeostatic Model Assessment of Insulin Resistance
HUVEC—Human umbilical vein endothelial cells

IFN-γ—Interferon-γ
iGLuc—IL-1β-Gaussia Luciferase fusion construct
IκBα—Inhibitor of kappa B
IKK—IκB kinase
IL-1β—Interleukin-1β
IL-18—Interleukin-18
iNOS—Inducible nitric-oxide synthase
IRE1-α —Inducible nitric-oxide synthase
JNK—Jun amino-terminal kinases
LDL—Low-density lipoprotein
LOX—Lipoxygenases
LPS--Lipopolysaccaride
LRR—Leucine-rich-repeat domain
MAM—Mitochondria-associated ER membrane
MAPK—Mitogen-activated protein kinases
MCP-1—Monocyte chemoattractant protein-1
MiR-223—MicroRNA-223
mtDNA—Mitochondrial DNA
MSU—Monosodium urate
MUFA—Monounsaturated fatty acids
NAC—N-acetyl-L-cysteine
NADPH—Nicotinamide adenine dinucleotide phosphate
NASH—Non-alcoholic steatohepatitis
NF-κB—Nuclear factor κB
NLRP3—NOD-like receptor family, pyrin domain containing 3
NO—Nitric oxide
oxLDL—Oxidized LDL
P2x7R—P2X purinoceptor 7
PAMP—Pathogen-associated molecular patterns PD1--
P-erk—Phospho-extracellular signal regulated kinase
PERK—Protein kinase R-like endoplasmic reticulum kinase
PPAR—Peroxisome proliferator-activated receptor
P-gly—P-glycoprotein
PRR—Pattern recognition receptors
PUFA—Polyunsaturated fatty acids
PVDF—Polyvinylidene difluoride
RIPA—Radioimmune Precipitation Assay
ROS—Reactive oxygen species
qPCR—Quantitative reverse transcription
RvD1—Resolvin D1
SFA—Saturated fatty acids
STZ—Streptozotocin
TAP—α-tocopherol associated protein
T2DM—Type 2 diabetes mellitus
TBST—Tris-buffered saline with tween-20
TLR—Toll-like receptor

TNF-α—Tumor necrosis factor α
TRF—Tocotrienol rich fraction
TRX—Thioredoxin
TUDCA—Tauroursodeoxycholic acid
TXNIP—Thioredoxin interacting protein
UFA—Unsaturated fatty acids
UPR—Unfolded protein response
VAT—Visceral adipose tissue
X/XO—Xanthine/xanthine oxidase

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