Regulation of NFκB-Mediated Inflammation By Green Tea
In Obese Models of Nonalcoholic Steatohepatitis

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
Jinhui Li, BM
Ohio State University Nutrition Graduate Program
The Ohio State University
2017

Dissertation Committee:
Richard S. Bruno, PhD, RD, Advisor
Amanda J. Bird, PhD
Ouliana Ziouzenkova, PhD
Andrea I. Doseff, PhD
Abstract

Green tea extract (GTE) protects against nuclear factor κB (NFκB)-mediated liver inflammation during nonalcoholic steatohepatitis (NASH). However, the mechanisms by which GTE exerts its antiinflammatory activities during NASH are unclear. There are several potential pathways and environmental conditions that mediate hepatic NFκB activation during NASH, including intracellular oxidative stress that is downregulated by nuclear factor E2-related factor 2 (Nrf2)-dependent antioxidant defenses, as well as the extracellular receptor-mediated pathways toll-like receptor 4 (TLR4) and tumor necrosis factor receptor 1 (TNFR1). Therefore, this dissertation aimed to define the involvement of these pathways in relation to antiinflammatory activities of GTE on hepatic NFκB activation during NASH. The central hypothesis was that GTE suppresses hepatic NFκB activation by increasing the activities of Nrf2, and by decreasing TLR4- and TNFR1-mediated signaling. Contrary to the hypothesis, studies in Nrf2-knockout mice demonstrated that GTE exerts its antiinflammatory activities against NFκB activation during NASH in an Nrf2-independent manner. Studies in wild-type mice that were fed with low-fat (LF) or high-fat (HF) diet containing 0 or 2% GTE demonstrated that GTE lowers NFκB-dependent inflammatory responses by reducing ligand availability for, and receptor expression of, TNFR1 and TLR4 pathways. Also, GTE protects against metabolic endotoxemia by restoring intestinal tight junction protein expression. Lastly, studies in loss-of-function TLR4-mutant mice showed that
GTE exerts its antiinflammatory activities at the liver in a TLR4-dependent manner, whereas intestinal-level benefits are mediated in a TLR4-independent manner. These findings are of significance in that they provide evidence to support GTE as a dietary strategy for NASH, specifically inflammation that mediates hepatic injury. These findings also provide basis for future clinical trials on the preventive and therapeutic potentials of GTE in mitigating liver inflammation during NASH.
Acknowledgments

I would like to express my sincerest gratitude to my advisor, Dr. Richard Bruno, for providing me the opportunity to conduct this research and numerous opportunities to learn new skills and collaborate with other researchers, for encouraging me to challenge myself academically, and for helping me with fellowship applications, abstracts, posters and oral presentations, and manuscript writing throughout the past 4 years of my Ph.D. study. I also would like to sincerely thank to other members of my committee, Dr. Amanda Bird and Dr. Ouliana Ziouzenkova, and Dr. Andrea Doseff for their time and advice from start to finish. This research could not have been done without all the collaborators, Dr. Philip Rohrer, Dr. José Manautou and Swetha Rudraiah. I would like to thank them for sharing their time and extensive knowledge and expertise.

I would like to thank my colleagues Teryn Sapper, Dr. Eunice Mah, Kevin Schill, Dr. Chureeporn Chitchumroonchokchai, Meredith Moller, Joshua McDonald, Yi Guo, Taylor Bahn, and Bradley Cotton for helping me with experiments. Without them, I would not have accomplished all the studies within years. I would like to especially thank Teryn, Eunice, and Chureeporn for the unwavering support, and mentorship, and friendship have been invaluable throughout my Ph.D. study. Additionally, I am grateful to have join OSUN and would like to thank to OSUN faculty and students, especially
Dr. Mark Failla, Dr. Jeffery Firkins, Dr. Rumana Yasmeen, Qiwen Shen, for their support and suggestions.

I would like to thank the organizations that contributed financially to support my education and research: The Graduate School University Fellowship, College of Education and Human Ecology (EHE) Dissertation Research Fellowships, EHE Scholarship; American Society for Nutrition (ASN) Graduate Student Research Award, American Society for Nutrition (ASN) ASN Emerging Leaders in Nutrition Science Award, Russell Klein Poster Competition Award, and travel awards from OSUN, EHE, and Department of Human Sciences.

Finally, I am extremely grateful for the friendship with Kevin E. Schill, Teryn N. Sapper, and Qiwen Shen. I would like to thank my parents Guoxin Li and Zhenfang Li, my aunt Jen Li and my uncle Jay Jiang, and two cousins Megan Jiang and Kaitlyn Jiang for countless encouragement and unconditional support.
Vita

2013 ........................................................................B.M., Preventive Medicine, Shandong University

2013 to present .....................................................Graduate Research Fellow, Department of Human Sciences, The Ohio State University

Publications

Research Publications

Li J, Sapper TN, Mah E, Moller MV, Kim JB, Chitchumroonchokchai C, McDonald JD, Bruno RS. *Green tea extract treatment reduces NFκB activation in mice with diet-induced nonalcoholic steatohepatitis by lowering TNFR1 and TLR4 expression and ligand availability.* J Nutr Biochem 2016 21;41:34-41.

Li J, Sapper TN, Mah E, Rudraiah S, Schill KE, Chitchumroonchokchai C, Moller MV, McDonald JD, Rohrer PR, Manautou JE, Bruno RS. *Green tea extract provides extensive Nrf2-independent protection against lipid accumulation and NFκB pro-inflammatory responses during nonalcoholic steatohepatitis in mice fed a high-fat diet.* Mol Nutr Food Res. 2016;60(4):858-70
Diamond S, Cotton BM, Banh T, Hsiao YH, Cole R, Li J, Simons C, Bruno RS, Belury MA, Vodovotz Y. *Raspberry ketone fails to reduce adiposity beyond decreasing food intake in C57BL/6 mice fed a high-fat diet.* Food Funct (Accepted)


**Abstracts**

**Li J, Chitchumroonchochhai C, Kim JB, Sasaki GY, Moller MV, Bouranis JA, Bruno RS.** Green tea extract protects against hepatic NFκB activation along the gut-liver axis in diet-induced obese mice with nonalcoholic steatohepatitis by reducing endotoxin and TLR4/MyD88 signaling. FASEB J. 2017 (Accepted)

**Li J, Chitchumroonchochhai C, Kim JB, Moller MV, Bruno RS.** Green tea extract lowers NFκB-mediated inflammation during nonalcoholic steatohepatitis in mice fed a high-fat diet consistent with reduced Toll-like receptor-4 signaling. FASEB J. 2016 30:269.2

**Li J, Sapper TN, Schill KE, Chitchumroonchochhai C, Moller MV, Rohrer PR, Rudraiah S, Manautou JE, Bruno RS.** Green tea extract attenuates oxidative stress,
inflammation, and lipogenesis through Nrf2-dependent and -independent mechanisms
in diet-induced obese mice with nonalcoholic steatohepatitis. FASEB J. 2015 29:390.4

Li J, Sapper TN, Schill KE, Chitchumroonchokchai C, Moller MV, Bruno RS. Green tea
extract attenuates NFκB-dependent inflammatory responses in diet-induced obese
mice with nonalcoholic steatohepatitis by lowering expression of TNFR1 and TLR4.
FASEB J. 2015 29:608.29

Fields of Study

Major Field: Ohio State University Nutrition Program
# Table of Contents

Abstract .............................................................................................................................. ii
Acknowledgments .............................................................................................................. iv
Vita ........................................................................................................................................ vi
Publications ........................................................................................................................ vi
Fields of Study .................................................................................................................... viii
List of Tables ........................................................................................................................ xiv
List of Figures ....................................................................................................................... xv

CHAPTER 1 ............................................................................................................................. 1
INTRODUCTION .................................................................................................................. 1
1.1. Overview ...................................................................................................................... 1
1.2. Central hypothesis and specific aims ......................................................................... 3

CHAPTER 2 ............................................................................................................................. 7
LITERATURE REVIEW ......................................................................................................... 7
2.1. The prevalence of NAFLD and NASH ................................................................. 7
2.2. The pathogenesis of NASH ....................................................................................... 8
  2.2.1. Insulin resistance and oxidative stress ......................................................... 9
  2.2.2. NFκB-mediated inflammation .................................................................. 11
  2.2.3. NASH pathogenesis along the gut-liver axis ............................................. 13
2.3. Green tea protects against NASH ........................................................................... 19
  2.3.1. Green tea is rich in catechins ................................................................... 19
  2.3.2. Green tea protects against “multiple-hits” of NASH .............................. 20
  2.3.3. GTE protects against NFκB activation at liver level .............................. 21
  2.3.4. Antiinflammatory activities of GTE against NFκB peripheral to the liver ... 27
2.4. Conclusion ................................................................................................................. 29

CHAPTER 3 ............................................................................................................................. 31
GREEN TEA EXTRACT PROVIDES EXTENSIVE NRF2-INDEPENDENT PROTECTION ON LIPID ACCUMULATION AND NFκB PRO-INFLAMMATORY
4.3. Materials and methods........................................................................................................ 65
  4.3.1. Materials .......................................................................................................................... 65
  4.3.2. Mouse study ....................................................................................................................... 65
  4.3.3. Histological Assessment of NASH .................................................................................. 67
  4.3.4. Metabolic Markers .......................................................................................................... 67
  4.3.5. Serum Endotoxin ............................................................................................................. 68
  4.3.6. RT-PCR .......................................................................................................................... 68
  4.3.7. Western Blotting ............................................................................................................. 69
  4.3.8. Statistical Analysis ......................................................................................................... 69

4.4. Results .................................................................................................................................. 70
  4.4.1. HF-feeding induces NASH ............................................................................................... 70
  4.4.2. GTE treatment ameliorates obesity, liver lipid accumulation, and insulin resistance without affecting energy intake .................................................................................. 70
  4.4.3. GTE treatment decreases histological and biochemical evidence of liver injury ................................................................................................................................. 71
  4.4.4. GTE ameliorates NFκB-mediated inflammation associated with NASH .................. 72
  4.4.5. GTE lowers ligand availability and expression of TNFR1 ........................................... 73
  4.4.6. GTE lowers TLR4 ligand availability and receptor complex expression ................. 74
  4.4.7. GTE increases intestinal TJP expression otherwise decreased by HF feeding ...... 75

4.5. Discussion ........................................................................................................................... 76

CHAPTER 5 .................................................................................................................................. 92
GREEN TEA EXTRACT PROTECTS AGAINST HEPATIC NFkB ACTIVATION ALONG THE GUT-LIVER AXIS IN DIET-INDUCED OBESE MICE WITH NONALCOHOLIC STEATOHEPATITIS BY REDUCING ENDOTOXIN AND TLR4/MYD88 SIGNALING .................................................................................................................. 92

5.1. Abstract .................................................................................................................................. 92
5.2. Introduction .......................................................................................................................... 93
5.3. Materials and methods ........................................................................................................ 95
  5.3.1. Materials .......................................................................................................................... 95
  5.3.2. Study design .................................................................................................................... 95
  5.3.3. Histological analysis of NASH ....................................................................................... 96
6.8.4. SFA-mediated TLR4/NFκB activation ................................................................. 139
6.8.5. GTE might suppress TLR4/MyD88/NFκB signaling by regulating other transcription factors ................................................................. 140
6.8.6. TLR4-dependent antiinflammatory activities through MyD88 and TRIF by GTE ................................................................. 141
6.9. Conclusion ............................................................................................................. 142
References ....................................................................................................................... 147
List of Tables

Table 1. Primers used for RT-PCR gene expression studies in Chapter 3 .......... 59

Table 2. Body composition, food intake, and serum and liver metabolic parameters in Nrf2-null and wild-type mice fed an HF diet containing GTE at 0% or 2% for 8 wk. .. 61

Table 3. Primers used for RT-PCR gene expression studies in Chapter 4............. 90

Table 4. Body composition, food intake, and serum and liver metabolic parameters in mice fed an LF or HF diet containing GTE at 0% or 2% for 8 wk. ......................... 91

Table 5. Primers used for RT-PCR gene expression studies in Chapter 5............ 123

Table 6. Body composition, insulin resistance, and liver lipid in WT and TLR4m mice fed a high-fat diet containing GTE at 0% or 2% for 8 wk................................. 124
List of Figures

Figure 1. The major catechins found in green tea (Camellia sinensis).......................... 6

Figure 2. Three candidate pathways that are likely involved in GTE-mediated protection against NFκB activation................................................................. 30

Figure 3. Time- and dose-response effects of EGCG on cell cytotoxicity and nuclear accumulation and mRNA expression of Nrf2 in HC-04 human hepatocytes. ......... 51

Figure 4. Hepatic mRNA expression of Nrf2 and Nqo1 in Nrf2-null and wild-type mice fed an HF diet containing GTE at 0 or 2% for 8 wk..................................................... 52

Figure 5. Histologic evaluation of livers from Nrf2-null and wild-type mice fed an HF diet containing GTE at 0 or 2% for 8 wk ................................................................. 53

Figure 6. Hepatic mRNA expression of genes involved in FFA uptake and lipid synthesis in Nrf2-null and wild-type mice fed an HF diet containing GTE at 0 or 2% for 8 wk................................................................. 54

Figure 7. Hepatic lipid peroxidation and antioxidant markers in Nrf2-null and wild-type mice fed an HF diet containing GTE at 0 or 2% for 8 wk ..................................................... 55

Figure 8. Hepatic protein of the phosphorylated p65 subunit of NFκB or total p65 in Nrf2-null and wild-type mice fed an HF diet containing GTE at 0 or 2% for 8 wk..... 56

Figure 9. Hepatic mRNA expression of pro-inflammatory genes in Nrf2-null and wild-type mice fed an HF diet containing GTE at 0 or 2% for 8 wk ............................... 57

Figure 10. Hepatic mRNA expression of pro-inflammatory genes in Nrf2-null and wild-type mice fed an HF diet containing GTE at 0 or 2% for 8 wk ............................... 58

Figure 11. Histological evaluation of hematoxylin and eosin-stained liver sections from mice fed an LF or HF diet for 12 wk ................................................................. 83

Figure 12. Histologic evaluation of livers from mice fed an LF or HF diet containing GTE at 0 or 2% for 8 wk ................................................................. 84

Figure 13. Protein expression of hepatic NFκB p65 and mRNA expression of hepatic pro-inflammatory genes in mice fed an LF or HF diet containing GTE at 0 or 2% for 8 wk ................................................................. 85
Figure 14. mRNA expression of TNFR1 complex genes and circulating concentrations of TNFα in mice fed an LF or HF diet containing GTE at 0 or 2% for 8 wk. 

Figure 15. Hepatic expression of TLR4 complex genes and circulating concentrations of endotoxin in mice fed an LF or HF diet containing GTE at 0 or 2% for 8 wk.

Figure 16. mRNA expression of small intestinal TJPs in mice fed an LF or HF diet containing GTE at 0 or 2% for 8 wk.

Figure 17. Hepatic protein expression of NFκB p65 and mRNA expression of NFκB-dependent pro-inflammatory genes in WT and TLR4m mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

Figure 18. Hepatic expression of TLR4 and its adaptor proteins MyD88 and TRIF in WT and TLR4m mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

Figure 19. Serum endotoxin and mRNA expression of small intestinal claudin-1 in WT and TLR4m mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

Figure 20. Serum liver injury, hepatic lipid peroxidation and glutathione redox status in WT and TLR4m mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

Figure 21. Histologic evaluation of livers from WT and TLR4m mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

Figure 22. Green tea extract protects against hepatic NFκB activation during NASH by inhibiting TLR4 and TNFR1 signaling independent of Nrf2-dependent antioxidant defenses.

Figure 23. MyD88- and TRIF-dependent TLR4 signaling that leads to hepatic NFκB activation during NASH.

Figure 24. GTE/EGCG might suppress TLR4/MyD88/NFκB signaling by regulating other transcription factors.
CHAPTER 1
INTRODUCTION

1.1. Overview

Nonalcoholic fatty liver disease (NAFLD) describes a spectrum of diseases starting from simple steatosis, and advances in severity to NASH, fibrosis, cirrhosis, and potentially hepatocellular carcinoma and liver failure [1]. NAFLD is the most prominent cause of liver disorder worldwide and in the United States [2]. It was estimated that 80-100 million individuals are afflicted [2]. NAFLD is defined as fatty hepatocytes occupying >5% of the hepatic parenchyma, without other causes for liver steatosis including significant alcohol consumption, viral hepatitis, medication use, or hereditary diseases [3, 4].

NASH, defined as liver steatosis in the presence of hepatocyte injury (i.e. ballooning and/or fibrosis) [3], is a progressive subtype of NAFLD. NASH has been identified as the leading cause of hepatocellular carcinoma, and the second indication for liver transplant in the United States after liver failure [5, 6]. The prevalence of NASH is associated with the prevalence of diabetes and metabolic syndrome [7]. Compared with simple steatosis, of which 1.5% patients progress to cirrhosis, 30-50% of NASH patients develop fibrosis, 15% to cirrhosis and 3% to terminal liver failure. NASH remains a severe, but reversible stage in the progression of NAFLD pathology. This highlights the need to develop effective preventive or therapeutic strategies of NASH.
that limit the risk of NAFLD. However, no validated treatment exists for NASH except for weight management, which has poor long-term success rate [8]. Thus, effective dietary strategies are required to treat and prevent NASH.

Green tea (*Camellia sinensis*) is one of the most widely consumed beverage in the world. Green tea and its catechins have been reported to improve NASH pathology in both preclinical [9] and human studies [10, 11]. The hepatoprotective effects of GTE are attributed to its catechins. There are four major green tea catechins: epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC), and epicatechin (EC) ([Figure 1] [12]). Among those, EGCG is the most abundant, accounting for 50-75% of the total catechin content of green tea. Chronic ingestion of green tea or tea catechins suppresses NASH in genetic- or diet-induced obese rodents [13-16].

Hepatic NFκB-mediated inflammation centrally regulates NASH pathogenesis by contributing to insulin resistance, liver steatosis, oxidative stress, and liver injury. GTE has been reported to protect against hepatic NFκB activation in rodent models of NASH in association with improved liver steatosis, injury and oxidative stress [16]. Thus, green tea and its catechins likely exerts their multifaceted protection against NASH through their antiinflammatory activities.

NFκB activation during NASH occurs in a redox-dependent manner due to overproduction of liver ROS [17, 18]. GTE increases hepatic glutathione, which is inversely correlated with hepatic NFκB binding activity [16]. GTE also attenuates NASH in genetically obese mice by lowering lipogenic gene expression and reducing lipid peroxidation in association with increased enzymatic activities of several Nrf2-dependent antioxidant defenses that detoxify ROS [15]. NFκB activation can also be
triggered by pro-inflammatory receptors, primarily TLR4 and TNFR1 signaling [17, 19, 20] (Figure 2). Lipopolysaccharide (LPS) functions as the ligand for TLR4 to activate NFκB-mediated inflammation. LPS-induced macrophage inducible nitric oxide synthase (iNOS) activity and expression is inhibited by green tea and its catechins in vitro [21-24]. GTE also decreases hepatic protein expression and adipose mRNA expression of tumor necrosis factor α (TNFα), the ligand for TNFR1, in ob/ob mice with NASH [15]. Thus, the protective effects for green tea catechins modulate each of these pathways (Nrf2, TLR4 and TNFR1).

1.2. Central hypothesis and specific aims

Building off existing evidence, the hypothesis of this dissertation is that GTE suppresses hepatic NFκB activation by increasing the activities of Nrf2, and by decreasing TLR4- and TNFR1-mediated signaling. Although the antiinflammatory effects of green tea and its catechins on diet- and genetic-induced NASH rodent models have been extensively studied, it is unclear how GTE exerts its antiinflammatory effects against NFκB activation during NASH. Chapter 3 defines the Nrf2-dependent and – independent antiinflammatory activities of GTE during NASH. Chapter 4 examines the effects of GTE on TNFR1 and TLR4 pathways that result in NFκB activation. Building off studies suggesting TLR4-dependent hepatoprotective activities of GTE, Chapter 5 defines the extent to which GTE-mediated antiinflammatory activities on NFκB activation are TLR4-dependent. Thus, the body of work presented in this dissertation addresses the below specific aims:

Aim 1. Define Nrf2-dependent antiinflammatory activities of GTE during NASH. GTE decreases NFκB activation in association with increased Nrf2-dependent cytoprotective antioxidant defenses [15]. However, whether the antiinflammatory
activities of GTE are mediated through Nrf2-dependent antioxidant activities has not been studied. The working hypothesis was that GTE protects against NFκB activation during NASH in an Nrf2-dependent manner to limit hepatic steatosis and oxidative stress. To test this, Nrf2-null and wild-type mice were fed an HF diet containing GTE prior to assessing NASH, Nrf2 expression, and NFκB-dependent inflammatory responses. Studies in vitro in Chapter 3 demonstrated that EGCG at physiologically relevant levels (0.1-5 μM) increased nuclear accumulation of Nrf2, indicating that EGCG, the major green tea catechin, activates Nrf2 in vitro. Consistent with studies in vitro, studies in vivo in Chapter 3 demonstrated that GTE upregulates hepatic mRNA expression of Nrf2 and its downstream gene NAD(P)H quinone oxidoreductase 1 (Nqo1) consistent with reduced oxidative stress-associated liver injury and NFκB activation. Although this potentials of green tea protect against NASH in an Nrf2-dependent manner, GTE was shown to attenuate hepatic NFκB activation and NASH in an Nrf2-independent manner. This supports that the antiinflammatory activities of GTE are not mediated through Nrf2-dependent antioxidant effects.

Aim 2. Define antiinflammatory activities of GTE against NASH occurring through TLR4 and TNFR1 signaling. TLR4 and TNFR1 are implicated as extracellular receptors that trigger NFκB-mediated inflammatory responses during NASH [17, 19, 20]. However, whether GTE reduces NFκB activation by downregulating TLR4- and TNFR1-mediated proinflammatory signaling has not been reported. The working hypothesis is that GTE inhibits NFκB activation during NASH by decreasing TLR4 and TNFR1 signaling. To test this, wild-type mice were fed an HF diet to induce NASH and subsequently provided GTE before assessing NASH, NFκB-dependent inflammation, and expression of TLR4 and TNFR1 receptor complexes and
their ligands. Studies in Chapter 4 demonstrated that GTE, when administered in a therapeutic manner, lowered hepatic inflammation and NASH. Data also demonstrated that GTE reduced NFκB-dependent inflammatory responses by lowering ligand availability and receptor expression of TNFR1 and TLR4 pathways. GTE also protects against metabolic endotoxemia consistent with a mechanism of restoring intestinal tight junction protein (TJP) expression.

**Aim 3. Define TLR4-dependent antiinflammatory activities of GTE.** Based on our published findings from Aims 1-2 [25, 26] showing that GTE decreases TLR4 expression, the *working hypothesis* is that GTE mitigates liver injury, oxidative stress, and NFκB-mediated inflammation during NASH in a TLR4-dependent manner. To test this, loss-of-function TLR4-mutant and wild-type mice were fed an HF diet containing GTE prior to evaluating NASH, NFκB activation, and TLR4 pathway. Studies in Chapter 5 demonstrated that GTE protects against hepatic NFκB activation along gut-liver axis in a TLR4-dependent manner at hepatic level, and in a TLR4-independent manner at intestinal level. GTE suppresses the TLR4/ myeloid differentiation protein 88 (MyD88) (MyD88) pathway that mediates hepatic NFκB activation without affecting TLR4/TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway regardless of genotype. This supports that GTE protects against NFκB activation in a TLR4/MyD88-dependent manner during NASH.
Figure 1. The major catechins found in green tea (*Camellia sinensis*).
CHAPTER 2
LITERATURE REVIEW

2.1. The prevalence of NAFLD and NASH

NAFLD describes a spectrum of liver disorders starting with relatively benign simple steatosis, and can progress to NASH, fibrosis, cirrhosis, and potentially liver failure or hepatocellular carcinoma [27]. NAFLD is now recognized as the leading cause of chronic liver disease [1] and is closely associated with obesity, insulin resistance, and/or metabolic syndrome [27]. NASH describes a relatively early stage of NAFLD with biochemical and histological evidence resembling alcoholic steatohepatitis [increased serum alanine aminotransferase (ALT), liver steatosis, and inflammatory infiltrates], but occurs in the absence of any history of alcohol abuse [28].

Despite being the “gold standard” for NAFLD diagnosis [29], the feasibility of liver biopsy is limited due to its high degree of invasiveness. Thus, imaging has become the most common diagnostic methodology for NAFLD, despite its likelihood of underestimating NAFLD compared with liver biopsy [30]. Other non-invasive methods including serum biochemical markers lack specificity and sensitivity, especially in evaluating hepatic inflammation and fibrosis [31]. Thus, reported NAFLD incidence varies based on the diagnostic method and is likely to be underestimated. The global incidence of NAFLD ranges from 6-33% with an estimated prevalence of 10-35% in Western European countries [32, 33]. In addition, 15–30 % of Asian adults and more
than 50% of diabetic patients are afflicted by NAFLD [34-37]. Consistent with obesity being a significant risk factor, estimates indicate that NAFLD afflicts 58-74% of obese adults and 23-53% of obese children [32, 38-41]. Moreover, its prevalence in the U.S. has increased dramatically in the past few decades, and the rapid increase in rates of NAFLD and NASH are congruent with the increased rates in obesity [42]. Thus, NAFLD is expected to currently affect 80-100 million Americans [2]. The prevalence of NAFLD is expected to increase due to both its close association with obesity and diabetes [43] and lack of validated therapies that mitigate its development and progression.

2.2. The pathogenesis of NASH

Although the underlying mechanism leading to NASH is not fully understood, its pathogenesis was originally described by the “two-hit” mechanism [44, 45]. This is supported by the observation that not all patients with simple steatosis progress to NASH [29]. The “first-hit” is mediated by obesity and insulin resistance, which results in dysregulated lipid metabolism and excessive liver lipid accumulation (simple steatosis). Simple steatosis is thought to predispose the liver to the “second-hit”, which is triggered by oxidative stress and NFκB-mediated inflammation [44]. However, the “two-hit” theory oversimplifies the etiology of NASH, and fails to consider lipotoxicity of free fatty acids (FFA), or how factors in the “second-hit” exacerbate the “first-hit”. Thus, several modified “two-hit” models and a “multiple-hit” model have been presented by the research community as alternatives.

Excess FFA, rather than triglyceride accumulation (steatosis), has been increasingly recognized as the reason for liver injury [46]. This leads to the modification of the “two-hit” theory to include the toxicity of FFA. Substantial evidence supports FFA-induced toxicity consistent with their induction of oxidative stress and inflammation [47].
In the modified “two-hit” mechanism, esterification of FFA with glycerol to synthesize triglyceride is viewed as protective against lipotoxicity [48]. Another extension of the traditional “two-hit” theory is that oxidative stress during the “second-hit” impairs hepatocyte proliferation to result in the accumulation of hepatocyte progenitors [49]. Both hepatocyte progenitors and hepatocyte-like cells that differentiate from the progenitors are associated with liver fibrosis and are implicated in hepatocellular carcinogenesis [49]. The traditional and modified “two-hit” mechanisms are also argued to be over-simplistic. Thus, the “multiple-hits” theory was proposed to explain the complexity of NASH pathogenesis [50] in which multiple events including insulin resistance, gut dysbiosis, and hepatic inflammation act in a concerted manner to induce NASH. NFκB-mediated inflammation is centrally implicated in the “multiple-hits” by exacerbating liver steatosis and oxidative stress-associated liver injury [51, 52]. This suggests that therapeutic strategies that limit NFκB activation would ameliorate NASH.

2.2.1. Insulin resistance and oxidative stress

Insulin resistance and compensatory hyperinsulinemia play an important role in promoting adipose-derived FFA flux to the liver. Insulin resistance blunts the inhibitory effects of insulin on hormone sensitive lipase, which provokes adipose lipolysis and release of FFA into circulation [53]. Insulin resistance also blocks the re-esterification of FFA into adipose tissue, which further contributes to the increased adipose-derived FFA flux to liver [54]. In addition to exacerbating insulin resistance to induce liver steatosis, an HF diet also directly contributes to liver steatosis because diet-derived FFA comprise 15% of the fatty acids that form liver triglyceride in NAFLD patients [55].

Liver steatosis results from increased FFA uptake into the liver, increased hepatic de novo lipogenesis and FFA esterification, and decreased triglyceride export from the
Hepatic FFA esterification to form triglyceride is dependent on hepatic fatty acid influx [57]. In addition, 26% of liver triglyceride content is derived from de novo lipogenesis in NAFLD patients [55] compared to <5% in healthy individuals [58, 59]. De novo lipogenesis increases due to insulin-mediated upregulation of lipogenic transcription factors, such as peroxisome proliferator activated receptor γ (PPARγ) and sterol regulatory element binding protein-1 (SREBP-1). Very low density lipoprotein (VLDL)-mediated triglyceride export from the liver is also impeded during NASH because of decreased apolipoprotein B (apoB) synthesis and lower activity of microsomal triglyceride transfer protein (MTP). ApoB and MTP are essential to incorporating triglyceride to apoB to form VLDL [60, 61].

Increased fatty acids from the diet and de novo lipogenesis activate PPARα [62, 63], which upregulates the expression of genes involved in mitochondrial fatty acid β-oxidation, such as carnitine palmitoyltransferase (CPT)-1 and CPT-2 [64]. In addition, FFA accumulation also induces lipotoxicity and results in mitochondrial dysfunction, leading to production of excessive reactive oxidative species (ROS). ROS derived from the mitochondria, peroxisomes and endoplasmic reticulum trigger lipid peroxidation of the mitochondrial membrane to induce ultrastructural lesions, loss of mitochondrial DNA, and impaired mitochondrial β-oxidation. NFκB-dependent proinflammatory cytokines promote insulin resistance, liver mitochondrial abnormalities, and ROS generation [51, 65]. This supports that therapeutic strategies that mitigate NFκB-mediated inflammation would ameliorate insulin resistance, liver mitochondrial abnormalities, and excessive ROS during NASH.
2.2.2. NFκB-mediated inflammation

2.2.2.1. NFκB is an inflammatory transcription factor

The transcription factor NFκB is a master regulator of inflammation [66]. NFκB is composed as either a heterodimer or homodimer of the Rel family including RelA (p65), RelB, c-Rel, p50 and p52 in humans [67]. The p65 subunit contains the most transactivation domains and is likely to be the strongest activator of NFκB-dependent genes [68]. Under normal conditions, NFκB is inactivated and sequestered in the cytoplasm due to its binding to the inhibitory protein IκB, which masks the nuclear localizing site of NFκB [69, 70]. Inflammatory signaling through extracellular receptors (TNFR and TLR) and intracellular ROS activate NFκB following the phosphorylation of IκB kinase (IKK), which in turn phosphorylates IκB and causes the dissociation of IκB from NFκB [71]. Following the release of NFκB from its inhibitor IκB, the p65 subunit can be phosphorylated by IKK at Ser468 and Ser536 residues to become transcriptionally active [72]. Pro-inflammatory signaling transduced by both TNFR1 [73] and TLR4 [74] can trigger the phosphorylation of the NFκB p65 subunit at Ser536.

2.2.2.2. NFκB activation plays an important role in the pathogenesis of NASH

Greater NFκB-mediated inflammation has been reported in obese patients with NASH [75], genetically obese (ob/ob) mice [76], and diet-induced obese mice with NASH [77, 78]. In contrast, suppressing liver NFκB activation ameliorates steatosis, oxidative stress, inflammatory responses and insulin resistance during NASH [79]. Heightened hepatic NFκB DNA binding activity in NASH patients is related to insulin resistance [75]. Conversely, inhibition of IKKβ, the upstream kinase functions to release NFκB from its inhibitor, thereby preventing NASH in mice fed a methionine and choline deficient (MCD) diet [79]. Hyperactivation of IKKβ in hepatocytes results in insulin
resistance, hyperlipidemia, and exacerbated liver inflammation in mice fed an HF diet [51]. This is consistent with rodent studies in which there is a genetic knockdown of IKKβ in hepatocytes and myeloid cells, which shows that IKKβ-mediated NFκB activation acts locally in liver and systemically in myeloid cells to cause insulin resistance [80]. It has also been reported that TNFα, an NFκB-dependent cytokine, promotes liver steatosis [81] and insulin resistance [82], and that inhibiting TNFα improves insulin sensitivity and liver steatosis [83-85]. Insulin resistance contributes to liver steatosis by increasing FFA flux from adipose tissue to liver [54] and amelioration of NFκB activation during NASH likely suppresses liver steatosis by improving insulin resistance.

NFκB-dependent proinflammatory enzymes promote the generation of ROS [86]. For example, NADPH oxidase (NOX) and cyclooxygenase-2 (COX-2) induce superoxide generation, while iNOS potentiates the generation of nitrogen dioxide radicals downstream of nitric oxide synthesis [86]. NFκB-induced oxidative stress promotes lipid peroxidation of mitochondrial membranes, impairment of the respiratory chain, and loss of mitochondrial DNA (mtDNA), resulting in mitochondrial dysfunction [87]. Conversely, mitochondrial dysfunction perpetuates ROS generation and lipid peroxidation [88]. NFκB-induced oxidative stress further exacerbates liver steatosis by increasing ApoB100 degradation, thereby inhibiting hepatic VLDL export [89].

NFκB also functions as the link between hepatic injury and fibrosis [19]. NFκB centrally regulates proinflammatory and anti-apoptotic signaling in the liver [19]. Increased inflammation or apoptosis results in liver injury. NFκB contributes to fibrogenesis by promoting liver injury and inflammatory signaling that triggers fibrogenic reactions in hepatic stellate cells and hepatic myofibroblasts that are derived from
hepatic stellate cells [90]. Increased phosphorylation of the NFκB p65 subunit at Ser536 has been found in human and mouse models of liver fibrosis [91]. Thus, NFκB-mediated inflammation not only contributes to liver steatosis, insulin resistance and oxidative stress during NASH, but accelerates the progression from NASH to fibrosis.

Collectively, NFκB-mediated inflammation is implicated in provoking insulin resistance and mitochondrial dysfunction leading to liver steatosis and oxidative stress during NASH [51]. That NFκB contributes to “multiple-hits” during NASH suggests that dietary strategies that target NFκB activation likely protect against NASH.

2.2.3. NASH pathogenesis along the gut-liver axis

2.2.3.1. Metabolic endotoxemia triggers TLR4-mediated hepatic inflammation during NASH

Metabolic endotoxemia, leaky gut, and gut bacterial dysbiosis plays an important role in NASH pathogenesis [92]. Metabolic endotoxemia is closely related to obesity and insulin resistance [93], which mediate the first-hit of NASH pathogenesis, and is defined by a two to three fold increase of serum lipopolysaccharide (LPS; endotoxin) [93]. LPS comprises ~75% of the cell wall of intestinal Gram-negative bacteria [94] and is a gut-derived ligand that triggers hepatic TLR4-mediated NFκB activation [95]. TLR4 mutant mice lacking intact TLR4/MyD88 signaling are protected from NASH that is otherwise induced by a high fructose diet despite increased portal endotoxin levels [96]. NASH patients have increased serum endotoxin, endotoxin core antibody (EndoCAb IgG), and higher expression of hepatic TLR4 [97, 98].

Gut-derived LPS is recognized at the liver by TLR4 and its two co-receptors [cluster of differentiation 14 (CD14) and myeloid differentiation protein (MD2)]. CD14 initially recognizes and binds circulating LPS, and LPS binding to CD14 is improved by
LPS binding protein. After binding to CD14, LPS is transferred to TLR4-MD2 heterodimers, which triggers intracellular inflammatory signaling to activate IKK and subsequent NFκB translocation to the nucleus.

The intracellular signaling of TLR4 is mediated through its two adaptor proteins MyD88 and TRIF, which regulate “early” and “late” NFκB activation, respectively [99]. MyD88-dependent signaling transduction is mediated directly by cell surface binding of LPS [100]. In contrast, TRIF-dependent signaling requires endocytosis of LPS [100], which serves to facilitate endotoxin clearance [101]. In addition to “late” NFκB activation, TLR4/TRIF signaling can also activate interferon regulatory factor 3 and 7, which upregulate the expression of interferon β (IFNβ) and further contributes to inflammation [99]. NFκB-dependent or IFNβ-dependent proinflammatory cytokines prolong hepatic inflammation, thereby exacerbating liver injury and accelerates liver fibrosis [102].

TLR4 is expressed not only in neutrophils and Kupffer cells, but in all other cells of the liver, including hepatocytes, stellate cells, sinusoid endothelial cells, and biliary epithelial cells [103]. This supports the investigation of hepatic inflammation, instead of the inflammatory responses mediated only by neutrophils or Kupffer cells. Thus, this dissertation investigated liver inflammation during NASH, to examine the overall responses mediated by TLR4 to gut-derived endotoxin.

Metabolic endotoxemia also exacerbates liver steatosis [104]. Rats administered with LPS have higher expression of lipogenic genes, such as SREBP-1c and fatty acid synthase (FAS). Metabolic endotoxemia is also associated with lower adipose-derived adiponectin, an important anti-lipogenic and antiinflammatory factor to prevent liver steatosis and inflammation during NASH pathogenesis [105]. In addition, LPS
administration impairs peroxisomal fatty acid β-oxidation [106] and further contributes to liver steatosis. Thus, it is likely that metabolic endotoxemia exacerbates liver steatosis during NASH through TLR4-mediated NFκB activation. This dissertation will examine the effect of metabolic endotoxemia on liver lipid metabolism in mice lacking intact TLR4 signaling.

2.2.3.2. Leaky gut contributes to metabolic endotoxemia during NASH

Both increased intestinal permeability and gut bacterial dysbiosis can cause metabolic endotoxemia. Intestinal permeability is tightly regulated by intestinal TJPs. TJPs are located at the lateral membrane of epithelial cells and control the intestinal paracellular transportation of small molecules and water. Intracellular plaque TJPs [zonula occluden (ZO) and cingulin] anchor integral transmembrane TJPs [claudin (CLDN), occludin (OCC), junctional adhesion molecule (JAM), and tricellulin] to the cytoskeleton to maintain gut barrier integrity [85]. Altered expression of intracellular or transmembrane TJPs as well as their post-translational modification and assembly are involved in the leaky gut. Impaired gut barrier function has been implicated in obesity [93] and NASH [107] due to excessive oxidative stress [108], proinflammatory cytokines [109], and overgrowth of intestinal pathogenic bacteria [110]. This indicates that dietary strategies targeting oxidative stress, inflammation and pathogenic gut bacteria likely mitigate NASH by restoring gut barrier function.

2.2.3.3. Gut bacterial dysbiosis contributes to endotoxemia during NASH

2.2.3.3.1. Small intestinal bacteria overgrowth and gut microbiota

Gut bacterial dysbiosis is closely related to insulin resistance [111] and endotoxemia-triggered hepatic inflammation [112], which mediates the “first-hit” and “second-hit” of NASH pathogenesis, respectively. Patients with metabolic syndrome
receiving transplantation of gut microbiota from healthy individuals have higher insulin sensitivity [113]. Gut bacterial dysbiosis includes small intestinal bacterial overgrowth (SIBO) and gut microbiota profile alteration, both of which are closely related to NASH pathogenesis.

SIBO plays an important role in obesity-related NAFLD [114] and occurs in more than 50% of NAFLD patients [92]. Healthy humans have about 10^{14} gut bacteria, including more than 200 species with a predominance of anaerobic bacteria. Gut dysbiosis contributes to NASH through endotoxemia-triggered NFκB activation. Increased intestinal bacterial load, especially Gram-negative bacteria, contributes to gut translocation of endotoxin into the bloodstream. In addition, SIBO also disrupts gut barrier function, resulting in leaky gut and endotoxemia. The gut-liver axis in NASH pathogenesis is supported by evidence that NASH patients have SIBO that correlates with plasma endotoxin levels and expression of TLR4, instead of TLR2 [115]. Antibiotics protect against metabolic endotoxemia and liver steatosis in rodent models of NASH induced by a high fructose diet [116], supporting that amelioration of SIBO mitigates NASH pathogenesis.

Alteration of the gut microbiota profile is also associated with obesity, insulin resistance, and NASH. The healthy human gut harbors two major bacterial phyla *Firmicutes* (Gram-positive; 60-80%) and *Bacteroidetes* (Gram-negative; 20-40%) [117]. Obese mice have distinct gut microbiota profile with predominantly *Firmicutes* with a decreased proportion of *Bacteroidetes* [118, 119]. Similarly, a NAFLD-prone phenotype presents more *Firmicutes* while NAFLD-resistant mice have more *Bacteroides* [120]. The predominance of *Firmicutes* is closely related to NASH pathogenesis by contributing to insulin resistance, liver steatosis, and increased mRNA
expression of genes involved in de novo lipogenesis regardless of obesity [121]. Increased proportions of *Firmicutes* in obese patients [122] promote fiber fermentation to produce acetate and thus increase energy harvesting from the diet [123]. Decreased *Bacteroidetes* in NASH patients likely contributes to NASH pathogenesis by limiting its competition with *Firmicutes* in energy and space to facilitate *Firmicutes* in dietary energy extraction [124]. Decreased *Bacteroidetes* in obese patients also occurs with increased *Actinobacteria*, which convert indigestible polysaccharides to monosaccharides and short-chain fatty acids. This results in increased energy extraction from the diet and increased hepatic lipid accumulation [125]. In addition, obese patients have more hydrogen (H₂)-producing bacteria (*Prevotellaceae*) and H₂-utilizing *Archaea* compared to controls with normal weight, or after gastric bypass surgery [123]. The H₂ transfer between H₂-producing bacteria and H₂-utilizing *Archaea* is associated with increased intestinal energy uptake [123]. Genetic-induced obese mice (*ob/ob*) with probiotic treatment have lower serum ALT, NFκB binding activity, and hepatic inflammation [85]. This supports that the amelioration of gut dysbiosis mitigates NASH pathogenesis.

### 2.2.3.3.2. Gut bacterial dysbiosis contributes to NASH

Similar to leaky gut, gut dysbiosis is closely related to liver NFκB activation during NASH by exacerbating metabolic endotoxemia. Hepatic TLR4 receptor recognizes gut-derived endotoxin and transduces inflammatory signaling to activate NFκB. SIBO and leaky gut both contribute to endotoxin gut translocation into the bloodstream and thus increase the ligand availability for hepatic TLR4.

Gut bacterial dysbiosis also exacerbates NASH by disrupting gastrointestinal hormone levels to increase energy harvest and fat storage. Gut microbiota can
increase energy-harvesting from the host’s diet [126]. Transplantation of gut microbiota from obese mice into germ-free mice induces obesity regardless of environmental factors [127]. Gut microbiota can also regulate glucose and lipid metabolism through enteroendocrine hormones, such as glucagon-like peptide 1 and peptide YY [128]. Glucagon-like peptide 1 promotes the secretion of insulin in pancreatic β-cells and increases cellular energy uptake/storage [129]. Peptide YY inhibits gut mobility and increases intestinal transit rate [130]. Gut dysbiosis results in higher fatty acid uptake and excessive fat accumulation in adipose tissue and liver by increasing lipoprotein lipase activity. Gut microbiota also contribute to dysregulated lipid metabolism by lowering the expression of fasting-induced adipose factor. Fasting-induced adipose factor acts as a suppressor of lipoprotein lipase. Thus, gut dysbiosis-mediated suppression of fasting-induced adipose factor increases adipose-derived fatty acid flow to the liver [126] and contributes to liver steatosis. Compared to germ-free mice, mice with gut microbiota had lower muscular and hepatic phosphorylated AMP-activated protein kinase, which plays an important role in stimulating fatty acid oxidation. Thus, gut dysbiosis contributes to obesity and liver steatosis by lowering fatty acid oxidation.

2.2.3.3. Gut bacterial dysbiosis is attributed to diet

Gut microbiota profile change is influenced by diet rather than obesity as evidenced by the correlation between gut microbiota change and carbohydrate uptake [131]. This is further supported by rodent studies showing that an HF diet, instead of obesity, increases Firmicutes [132], indicating the potential of dietary therapy in altering gut microbiota towards that of lean adults.

The mechanisms by which dietary patterns cause gut microbiota profile change, SIBO, and impaired gut barrier function is not fully understood. However, it has been
found that HF-induced insulin resistance decreases gastrointestinal motility and causes the retention of food content in gastrointestinal lumen to favor SIBO [93, 133]. Insulin resistance also impedes intestinal pH-lowering effects due to bacterial fermentation of carbohydrate [134]. The resulting neutral pH favors the overgrowth of pathogenic bacteria while inhibiting beneficial bacteria such as butyrate producers. Butyrate and other short chain fatty acids are the major intestinal fuel source for gut bacteria and important in maintaining/regulating TJPs and mucins. This is supported by evidence showing that implantation of butyrate-producing C. butyricum mitigates liver steatosis, injury and hepatic inflammation in rats models of NASH [135]. Improved NASH parameters are associated with lower metabolic endotoxemia and higher expression of intestinal TJPs including ZO and OCC [135]. The fact that dietary patterns alter gut microbiota support dietary strategies to mitigate NASH by improving gut health. Since gut microbiota and associated gut barrier function is regulated by diet, dietary intake of prebiotic supplements, such as GTE [136], might improve gut barrier function to protect against metabolic endotoxemia and liver inflammation during NASH.

2.3. Green tea protects against NASH

2.3.1. Green tea is rich in catechins

Tea is one of the most widely consumed beverages in the world after water [137]. Unlike fermented tea (black tea and oolong tea), catechins (flavan-3-ols) in green tea are well-preserved and constitute 30-42% of the solid weight of brewed green tea. During post-harvest withering and steaming, green tea catechins are preserved due to rapid inactivation of polyphenol oxidase [138]. Four major green tea catechins are EGCG, ECG, EGC, and EC [12]. EGCG is the most abundant green tea catechin, which accounts for 50-75% of the total catechin content.
Epidemiological studies show that high intakes of green tea (≥10 cups/d) lower the risk for liver injury and inflammation in NASH patients [139]. Recent clinical studies demonstrate that GTE also treats liver steatosis and improves liver function tests [10, 11]. GTE protects against NASH in genetic and diet-induced obese rodent models through hypolipidemic, antioxidant, and anti-inflammatory mechanisms [13-16].

2.3.2. Green tea protects against “multiple-hits” of NASH

Liver steatosis is caused by increased FFA flux to the liver [54], disruption of β-oxidation to remove excess FFA or lipid export in VLDL [44], and upregulated liver lipogenesis [140]. Green tea inhibits intestinal lipid absorption by lowering pancreatic phospholipase A(2) activity [141]. Green tea catechins also ameliorate liver steatosis and injury by decreasing lipogenic gene expression [142], and increasing the expression of fatty acid oxidation genes [143]. Multiple studies demonstrate that GTE protects against the “first-hit” of NASH by inhibiting liver steatosis in genetic- [13-15] and HF diet-induced rodent models of NASH [16].

The “second-hit” is induced by oxidative stress and NFκB-mediated inflammation. GTE inhibits lipid peroxidation by limiting NADPH oxidase activity, and that GTE decreases protein nitration by reducing iNOS expression which is otherwise increased during NASH [13]. GTE also exerts antioxidant activity by restoring hepatic glutathione, and increasing enzymatic activities of Nrf2-dependent endogenous antioxidant defenses [superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione-S-transferase] [16]. In HF diet-induced rodent models of NASH, GTE lowers NFκB binding activity and expression of NFκB-mediated inflammatory genes, TNFα, MCP-1 [16], and COX-2 [144].
2.3.3. GTE protects against NFκB activation at liver level

2.3.3.1. GTE likely suppresses NFκB activation through its antioxidant activities

NFκB activation during NASH can be triggered by oxidative stress due to overproduction of liver ROS [17, 18] (Figure 2). HF feeding increases liver ROS generation by inhibiting mitochondrial and peroxisomal fatty acid oxidation and mitochondrial oxidative-phosphorylation [145]. ROS contributes to insulin resistance [146] and limits hepatic VLDL export of triglycerides [89], which collectively exacerbate liver lipid accumulation during NASH. Hepatic NFκB DNA binding activity is negatively correlated with serum antioxidant capacity in obese patients [75].

2.3.3.1.1. Nrf2-dependent antioxidant activities of GTE

Nrf2 is an antioxidant transcription factor that binds to antioxidant response elements and controls the expression of heme oxygenase-1, Nqo1, and GCLc, which play a pivotal role in intracellular ROS scavenging [147]. Nrf2-dependent antioxidant defenses are capable of inhibiting NFκB activation and NASH progression. The pharmacological Nrf2 activator Protandim increases SOD and catalase activity by 30-54% in human erythrocytes [148], indicating its potential to increase ROS detoxification to mitigate NASH. The overexpression of Nrf2-dependent enzymes heme oxygenase-1 and Nqo1 in human monocytes, prevents LPS-induced expression of TNFα, which is an NFκB-dependent inflammatory cytokine [149]. Heme oxygenase-1 has also been found to inhibit the phosphorylation of NFκB p65 subunit [150]. Nrf2 activation by synthetic triterpenoid 1[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole prevents HF-induced obesity and NASH [151]. Nrf2 also exerts anti-fibrotic effects in hepatic stellate cells by inhibiting transforming growth factor β-mediated pro-fibrotic signaling and stellate cell activation [152, 153]. Consistently, the inhibition/absence of
Nrf2-dependent antioxidant defenses exacerbates liver inflammation during NASH by increasing the expression of hepatic NFκB downstream genes [154]. Nrf2 knockout mice have accelerated onset and progression of NASH [155]. Thus, Nrf2 has been considered as a target for NASH therapy [156].

A study in vitro shows that EGCG exerts direct antioxidant effects to scavenge ROS. However, low bioavailability of catechins in vivo limits their potentials to directly scavenge ROS. Catechins are able to upregulate Nrf2-dependent antioxidant defenses [15]. This is important because Nrf2-dependent antioxidant defenses are also expected to reduce ROS-mediated NFκB activation. GTE increases hepatic glutathione, which was inversely correlated to hepatic NFκB binding activity [16]. GTE treatment also increases mRNA expression of the glutamate-cysteine ligase catalytic subunit (GCLc), which is not only the rate limiting enzyme for glutathione biosynthesis, but is also a downstream gene of Nrf2 [16]. GTE also attenuates NASH in genetically obese mice by lowering lipogenic gene expression and reducing lipid peroxidation in association with increased enzymatic activities of several Nrf2-dependent enzymatic antioxidant defenses that detoxify ROS [15]. This is further supported by the evidence showing that GTE supplementation upregulates hepatic Nrf2 nuclear translocation as well as the expression of Nrf2-dependent antioxidants in mice treated with polychlorinated biphenyls-induced oxidative stress [157].

2.3.3.1.2. EGCG increases Nrf2 nuclear translocation

Nrf2 activation is regulated by a “hinge and latch” mechanism [158, 159]. Under homeostatic conditions, Nrf2 binds to its inhibitory protein Keap1 and constitutively undergoes ubiquitination degradation. Green tea catechins exert antioxidant effects through their electrophilic potential [160]. Catechins are readily auto-oxidized at near-
neutral pH in cell culture or during digestion [161] or by intracellular ROS. Oxidized catechins trigger oxidative modification of cysteine residues on Keap1 to induce conformational changes from two-site recognition to one-site binding. The conformational change of Keap1-Nrf2 impedes the ubiquitination degradation of Nrf2. Excessive cytosolic Nrf2 due to the inhibition of ubiquitination degradation facilitates Nrf2 nuclear translocation to maintain the balance between cytosolic and nuclear Nrf2. When translocated to the nucleus, Nrf2 binds to antioxidant response elements to upregulate the expression of antioxidant genes [158, 159].

Although EGCG induces Nrf2 nuclear translocation in vitro and GTE upregulates mRNA expression of hepatic Nrf2-dependent antioxidants in vivo, whether or not GTE exerts its antiinflammatory activities against NFκB activation in an Nrf2-independent manner has not been investigated. Studies in Chapter 3 will examine the Nrf2-dependent protection against NFκB activation during NASH by GTE [25]. Chapter 3 examines the antioxidant and antiinflammatory activities of GTE, as well as the relationship between the Nrf2-dependent antioxidant and antiinflammatory activities by GTE. This will fill the gap in knowledge regarding the role of Nrf2 in GTE-mediated protection against NFκB activation.

2.3.3.1.3. Nrf2-independent antioxidant activities of GTE

Although Nrf2 is the major regulator of intracellular redox status, other antioxidant transcription factors, such as forkhead box, class O (FoxO) and Nrf1, are also involved in the antioxidant activities of green tea catechins during NASH. Their potential to mediate the antiinflammatory activities of GTE against NFκB activation warrants investigation. FoxO transcriptionally regulates the expression of mitochondrial antioxidants (SOD-2, peroxiredoxins 3 and 5), peroxisomal catalase, and plasma...
selenoprotein P and ceruloplasmin. Intracellular ROS activates FoxO by transcriptionally upregulating FoxO mRNA expression, post-translationally regulating FoxO phosphorylation and acetylation, and changing FoxO subcellular localization [162]. NASH patients have increased expression and altered subcellular localization of FoxO [163]. EGCG mitigate endothelin-1 expression in vascular epithelial cells [164]. FoxO3a has also been found to mediate hepatocyte lipo-apoptosis and centrally regulates SOD-2 expression during NASH [165]. EGCG has been reported to activate FoxO3a in breast cancer cells [166].

Likewise, Nrf1-dependent hepatoprotection due to GTE supplementation likely mediates GTE antiinflammatory activities against NFκB. Similar to Nrf2, Nrf1 also binds to antioxidant response elements to regulate antioxidant activities and lipid metabolism [47]. Unlike Nrf2 which provides secondary defense against unscheduled stress during NASH, Nrf1 limits steady-state cellular stress and prevents the generation of excessive ROS [47]. Indeed, mice with liver-specific Nrf1 knockout have dysregulated hepatic lipid metabolism [48], which mediates the “first-hit” of NASH and contributes to NFκB-mediated inflammatory responses. This occurs consistent with Nrf1 regulating the expression of PPARα and PPARγ coactivators [49]. Thus, GTE likely mitigates liver steatosis and oxidative stress during NASH through an Nrf1-dependent manner. This is further supported by the evidence that EGCG prevents excessive liver lipid accumulation and injury in HF-fed mice while upregulating skeletal muscle mRNA expression of Nrf1 and fatty acid oxidation genes [50]. GTE likely exerts its antioxidant and hypolipidemic activities by activating several antioxidant transcription factors (i.e. Nrf2, FoxO, Sirtuin, and Nrf1).
2.3.3.2. Green tea protects against TNFR1/NFκB activation

NFκB activation can also be triggered by TNFR1 and TLR4 that mediate pro-inflammatory signaling pathways [17, 19, 20] (Figure 2). Antibodies to TNF also inhibit NFκB-mediated inflammatory responses to protect against NASH in ob/ob mice [85]. TNFα is not only a pro-inflammatory cytokine regulated by NFκB, but an extracellular stimuli for NFκB activation during NASH in mice with fructose-induced NASH [167]. TNFα binds to two distinct cell-surface receptors, TNFR1 and TNFR2. TNFα has higher affinity for TNFR1. Thus, TNFR1 is considered the major TNFα receptor during NASH. TNFR1 deficiency prevents NASH [167], and TNFR1 gain-of-function mutation exacerbates NASH in mice fed an HF diet [168]. NASH patients also have higher expression of TNFR1 and its ligand TNFα [169]. It has been elucidated that TNFα-TNFR1 binding recruits adaptor protein TNFR-associated death domain (TRADD) and receptor interacting protein 1 (RIP1) to form a functional TNFR1 signaling complex which facilitates NFκB phosphorylation and nuclear translocation [170]. GTE decreases hepatic protein expression and adipose mRNA expression of TNFα, the ligand for TNFR1, in ob/ob mice with NASH [15]. EGCG inhibits the formation of TNFR1 receptor complex in mice with TNFα-mediated lung inflammation [171]. Evidence in vitro also shows that EGCG inhibits TNFR1 protein expression during TNFα-induced inflammation [172].

GTE protects against hepatic NFκB-mediated inflammation during NASH. GTE and EGCG have been reported to lower TNFα in ob/ob mice [15] and TNFR1 expression in vitro [172]. However, whether GTE exerts its antiinflammatory activities by lowering TNFR1 signaling is not understood. Thus, studies in Chapter 4 will examine
the effects of GTE on receptor expression and ligand availability of the TNFR1 pathway during NASH.

### 2.3.3.3. Green tea protects against TLR4/NFκB activation

Endotoxemia caused by SIBO and increased intestinal permeability contributes to hepatic NFκB activation in NASH patients [92]. NASH patients have increased plasma endotoxin, which induces TLR4 signaling and nuclear translocation of NFκB [173]. Despite high portal endotoxin levels, TLR4-mutant mice fed a high-fructose diet are protected from hepatic NFκB-mediated TNFα expression and NASH pathology [96]. Antibiotics protect against liver steatosis in fructose-fed mice by ameliorating endotoxemia [116]. The TLR4 receptor complex located on the cell plasma membrane is comprised of TLR4, its co-receptors (CD14 and MD2) and adaptor protein MyD88 [174]. LPS-induced iNOS activity and expression in macrophages is inhibited by green tea and its catechins [21-24]. Green tea consumption also improves human gut health by increasing the proportion of *Bifidobacterium* [175], an important probiotic species that can improve gut barrier function and thus reduce intestinal translocation of LPS [176].

GTE has been reported to improve gut health and protect against hepatic NFκB-mediated inflammation during NASH. However, whether GTE exerts its antiinflammatory activities by lowering TLR4 signaling is not fully understood. Studies in Chapter 4 will examine the effects of GTE on mRNA expression of the TLR4-MD2-CD14-MyD88 receptor complex when administered in a therapeutic manner. Furthermore, levels of circulating endotoxemia and gut TJP expression will be measured to evaluate the gut barrier integrity during NASH. MyD88 and TRIF are two adaptor proteins that transduce TLR4 signaling to NFκB. Studies in Chapter 5 will
examine MyD88 and TRIF expression to evaluate the effects of GTE on TLR4/NFκB activation that is mediated by MyD88 and TRIF during NASH.

In addition to targeting ligand availability and receptor expression of LPS/TLR4 pathway, GTE and its catechins might also target the TLR4 inhibitory pathway, or saturated fatty acids (SFA)/TLR4 pathway. Evidence in vitro shows that EGCG rapidly upregulates Tollip, an inhibitor of TLR4 signaling, by interacting with the 67LR [177]. GTE was also shown to lower NADPH oxidase activity and peroxynitrite-mediated damage in obese mice with NASH [13]. This supports the possibility that GTE inhibits TLR4 trafficking and assembly, which requires peroxynitrite that is generated downstream of NADPH oxidase activation [178]. Furthermore, SFA, especially palmitate (C16:0), also activate TLR4-mediated pro-inflammatory signaling [179]. Both adipose-derived and HF diet-mediated increases in FFA have high levels of SFA. GTE likely exerts its antiinflammatory activities against TLR4/NFκB through its hypolipidemic effects by inhibiting adipose-derived FFA flux to liver.

Furthermore, green tea catechin metabolites formed in gut are likely to be involved in GTE-mediated antiinflammatory activities against TLR4/NFκB. Catechin metabolites Hydroxyphenyl-valerolactones, which are catechin metabolites, are associated with reduced COX-2-dependent eicosanoid accumulation in the human skin [180]. Hepatic COX-2 inflammatory signaling contributes to NASH pathogenesis, and thus GTE metabolites likely protect against hepatic COX-2 inflammation.

2.3.4. Antiinflammatory activities of GTE against NFκB peripheral to the liver

Despite being low in catechins compared to unfermented green tea, fermented green tea improves NASH symptoms by modifying gut microbiota composition, especially by decreasing the ratios of Firmicutes/Bacteroidetes, and
The *Firmicutes* phylum has been found to be the predominant bacterial composition in obese mice, and thus decreasing the composition of *Firmicutes* by fermented green tea helps restore gut microbiota in obese mice similar to that of healthy mice. In addition, lower *Bacteroides/Prevotella* ratios suggest carbohydrate fermentation and improved gut barrier function. Thus, fermented green tea improves gut microbiota composition for healthier gut function despite the fact that fermented green tea has only 14.1% catechins compared to 30% in unfermented green tea.

Polyphenols, such as green tea catechins, can be metabolized by gut microbiota. The cleavage of polyphenol glycosidic linkages generates glycans to favor the growth of *Bacteroidetes* instead of *Firmicutes* because *Bacteroidetes* is better at degrading glycan to harvest energy compared to *Firmicutes*. Since NASH patients have increased *Firmicutes* and decreased *Bacteroidetes*, restoring *Bacteroidetes/Firmicutes* composition in gut microbiota by catechins likely mitigates NASH. Furthermore, green tea catechins inhibit the growth of pathogenic bacteria such as *Clostridium difficile* and *Staphylococcus spp.* while contributing to the growth of beneficial bacteria *Bifidobacterium spp.*.

EGCG reverses IFNγ-induced impairments in intestinal barrier function *in vitro*. Recent study shows that EGCG improves epithelial barrier function by inducing the production of antimicrobial peptides in porcine intestinal epithelial cell monolayers. However, whether GTE improves gut barrier function to lower the translocation of gut-derived endotoxin to blood remains unknown. Thus, studies in Chapter 4 and 5 will examine the effects of GTE on the expression of TJPs and the levels of blood...
endotoxin to evaluate the effects of GTE on gut permeability. Studies in Chapter 5 will investigate whether GTE regulates gut permeability in a TLR4-dependent manner.

2.4. Conclusion

GTE protects against hepatic NFκB-mediated inflammation during NASH. Several candidate pathways likely mediate the antiinflammatory activities of GTE during NASH: Nrf2, TNFR1 and TLR4 (Figure 2). This dissertation will examine each pathway to evaluate whether GTE exerts its antiinflammatory activities by upregulating Nrf2-dependent antioxidant defenses, or by lowering TNFR1 or TLR4 signaling.
Figure 2. Three candidate pathways that are likely involved in GTE-mediated protection against NFκB activation.
CHAPTER 3
GREEN TEA EXTRACT PROVIDES EXTENSIVE NRF2-INDEPENDENT PROTECTION ON LIPID ACCUMULATION AND NFκB PRO-INFLAMMATORY RESPONSES DURING NONALCOHOLIC STEATOHEPATITIS IN MICE FED A HIGH-FAT DIET

3.1. Abstract

GTE reduces liver steatosis and inflammation during NASH. We hypothesized GTE would mitigate NASH in an Nrf2-dependent manner in an HF-induced model. Nrf2-null and WT mice were fed an HF diet containing 0 or 2% GTE for 8 wk prior to assessing parameters of NASH. Compared to WT mice, Nrf2-null mice had increased serum ALT, hepatic triglyceride, expression of FFA uptake and lipogenic genes, malondialdehyde (MDA), and NFκB phosphorylation and expression of pro-inflammatory genes. In WT mice, GTE increased Nrf2 and Nqo1 mRNA, and lowered hepatic steatosis, lipid uptake and lipogenic gene expression, MDA, and NFκB-dependent inflammation. In Nrf2-null mice, GTE lowered NFκB phosphorylation and TNFα and MCP1 mRNA to levels observed in WT mice fed GTE whereas hepatic triglyceride and lipogenic genes were lowered only to those of WT mice fed no GTE. MDA was lowered in Nrf2-null mice fed GTE, but not to levels of WT mice, and without improving the hepatic antioxidants α-tocopherol, ascorbic acid, and uric acid. Nrf2
deficiency exacerbates NASH whereas anti-inflammatory and hypolipidemic activities of GTE likely occur largely independent of Nrf2 signaling.

3.2. Introduction

NAFLD is the most common liver disorder in the United States [27]. It is a progressive disorder that advances in severity from liver steatosis to NASH, fibrosis, and cirrhosis to increase hepatocellular carcinoma risk [187]. An estimated 80-100 million Americans have NAFLD [2], and its prevalence is expected to increase due to its close association with obesity and diabetes [43] and a lack of validated therapies that mitigate its development and progression.

The pathogenesis from steatosis to NASH is often described by the “two-hit” mechanism [188]. The “first-hit”, mediated by obesity, insulin resistance, and dysregulated lipid metabolism, results in excess hepatic lipid accumulation [188]. Consequently, steatotic livers are vulnerable to “second-hits”, mediated by oxidative stress and inflammation, that exacerbate liver injury [188]. The involvement of reactive oxygen species (ROS) during NASH [188] supports a role of Nrf2 in limiting oxidative stress that otherwise induces liver injury [156]. Indeed, transcriptional activities of Nrf2 regulate xenobiotic metabolism and antioxidant defenses [156]. They also reduce inflammation and fatty acid synthesis [189-192], suggesting that its cytoprotective activities could attenuate NASH through multiple mechanisms.

Nrf2-dependent hepatoprotection has been demonstrated in several models of NASH. Nrf2-null mice fed a MCD diet had exacerbated pathology of NASH, including greater expression of pro-inflammatory and fatty acid metabolism genes as well as histological and biochemical markers of liver injury and lipid peroxidation [155]. Conversely, Kelch-like ECH-associated protein 1 (Keap1) knockout mice fed a MCD
diet are protected from NASH due to their constitutive activation of Nrf2 [193]. Nrf2-null mice fed an HF diet also show that Nrf2 reduces steatosis and oxidative stress by suppressing lipogenic and cholesterogenic pathways [194]. Nrf2 may also mitigate oxidative stress during NASH by improving redox status, consistent with its role in regulating glutathione biosynthesis and expression of antioxidant defenses (e.g. Nqo1) [156].

NASH patients and rodent models of NASH have greater NFκB activation and expression of pro-inflammatory genes including TNFα and monocyte chemoattractant protein-1 (MCP-1) [75, 77]. NFκB activation occurs through intracellular and extracellular pathways that converge at the phosphorylation of IKK, which promotes IκB phosphorylation and nuclear translocation of NFκB [195]. Nrf2 deficiency increases NFκB activation during NASH [154], consistent with evidence from a pro-inflammatory experimental model that Nrf2 activation decreases IKK activity and phosphorylation and degradation of IκBα [196]. Thus, therapies that induce Nrf2 would be expected to attenuate NFκB-dependent pro-inflammatory responses otherwise promoting liver injury during NASH.

High dietary intakes of green tea are associated with a lower risk of liver injury, hyperlipidemia, and inflammation [139]. Although no clinical studies have examined green tea during NASH, GTE protects against NASH, likely through its major catechin EGCG, in genetic and diet-induced models through hypolipidemic, antioxidant, and anti-inflammatory mechanisms [9]. For example, GTE attenuated NASH in genetically obese mice by lowering lipogenic gene expression and reducing lipid peroxidation in association with an upregulation in enzymatic activities of several Nrf2-dependent enzymatic antioxidant defenses that detoxify ROS [15]. In a dietary HF-induced model
of NASH, GTE lowered NFκB binding activity by reducing IkBα phosphorylation while also decreasing protein and mRNA levels of TNFα and MCP-1 [16]. Consistent with these findings and evidence that GTE improves endogenous antioxidant defenses under the regulation of Nrf2 [15], we hypothesized in the present study that hepatoprotective activities of GTE during NASH are mediated in an Nrf2-dependent manner. We therefore fed Nrf2-null mice and their wild-type (WT) controls an HF diet containing GTE at 0 or 2%, and then assessed markers of liver injury and oxidative stress, expression of genes involved in fatty acid uptake and lipogenesis, and NFκB-dependent inflammatory responses to define Nrf2-dependent hypolipidemic and anti-inflammatory activities of GTE during NASH.

3.3. Materials and Methods

3.3.1. Materials

All solvents were HPLC-grade or higher and were purchased from Thermo Scientific (Waltham, MA) along with the following chemicals: citric acid, EDTA, and PBS. BSA, EGCG, thiobarbituric acid (TBA), Tris-buffered saline with Tween-20 (TBST), and 1,1,3,3-tetramethoxypropane (TPP) were from Sigma (St. Louis, MO).

3.3.2. In Vitro Study

HC-04 human hepatocytes, a cell line well-suited for biomechanistic studies of xenobiotics [197], were used to examine dose- and time-dependent effects of EGCG, the predominant polyphenolic catechin in green tea [9], on Nrf2 activation and expression. HC-04 cells were obtained from BEI Resources (American Type Culture Collection; Manassas, VA). In all experiments, cells were incubated (5% CO₂; 37°C) in low glucose Dulbecco’s Modified Eagle Medium (DMEM; Sigma) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (100 U/mL penicillin G sodium,
100 µg/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B). EGCG at physiologically relevant concentrations (0-5 µM) [9] was dissolved in 0.05% citric acid and all experiments were performed in serum-free DMEM containing 1% antibiotic-antimycotic. Cells were seeded at a density of 4x10⁵ for mRNA expression studies or 8x10⁵ for all other studies and were harvested at specific time points up to 2 h following incubation with EGCG. Nuclear accumulation of Nrf2 protein in response to EGCG (0-5 µM) or the pro-oxidant positive control t-butylhydroperoxide (100 µM) was assessed by Western Blot, as described below, after isolating the nuclear fraction using a kit (Active Motif, Carlsbad, CA). Nrf2 mRNA expression was assessed at 0-5 µM EGCG at 0-2 h post-treatment using RT-PCR, as described below, after total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). Cytotoxicity was assessed using the lactate dehydrogenase (LDH) assay (Sigma) and the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Promega, Madison, WI) assay at 2 h post-treatment according to the manufacturers’ instructions.

3.3.3. Mouse Study

The protocol for this study was approved by the Institutional Animal Care and Use Committee (2012A00000156) at The Ohio State University. Homozygous Nrf2-null mice on a C57BL/6 background provided by Dr. Angela Slitt (University of Rhode Island) were bred at University of Connecticut, and then male Nrf2-null mice (n = 23) were supplied to The Ohio State University. Male C57BL6 WT mice (n = 20) were purchased from Charles River Laboratories (Wilmington, MA). All mice were acclimated to the temperature-, light-, and humidity-controlled facility for at least 2 wk until WT and Nrf2-null mice were 11-12 wk old. Nrf2-null and WT mice were housed individually while receiving an HF diet containing powdered GTE at 0 or 2% (w/w) for 8 wk. The HF diet
containing 60% of energy from fat (primarily as lard), as detailed previously [16, 198], was formulated to contain 0 or 2% GTE, which was kindly provided by Unilever BestFoods.

Powdered GTE contained 30% total catechins (w/w), which consisted of 48% EGCG, 31% EGC, 13% ECG, and 8% EC as verified by HPLC-UV [14]. GTE at 2% was chosen based on our studies showing that it reduces liver steatosis and NFκB-dependent inflammation in genetic and HF-induced models of NASH [14-16]. GTE at 2% is also closely equivalent to 10 servings/d (120 mL/serving) in humans [14], a dietary level associated with a lower risk of liver injury in Japanese adults [139]. Mice had free access to food and water throughout the study. Body mass was measured weekly and food intake daily. After the feeding period, mice were sacrificed in the fasted state (10-12 h) under isoflurane. Blood was collected from the retroorbital sinus and centrifuged to obtain serum. Tissues were excised, rinsed in PBS, blotted, and frozen in liquid nitrogen before storing at -80°C. A portion of the central hepatic lobe was collected into formalin for histological assessment or into RNAlater (Sigma) for gene expression studies.

3.3.4. Liver Histology

Paraffin-embedded liver sections (4-5 μm) were stained with hematoxylin and eosin. Images were captured using an Olympus IX50 microscope from 10 randomly selected fields (200x magnification) to assess NASH using established histologic criteria that score steatosis, hepatocellular ballooning, and lobular inflammation [199]. In brief, steatosis was scored as: grade 0 for no fatty hepatocytes, grade 1 for fatty hepatocytes occupying <33% of the hepatic parenchyma, grade 2 for fatty hepatocytes occupying 33-66% of the hepatic parenchyma, or grade 3 for fatty hepatocytes...
occupying >66% of the hepatic parenchyma. Hepatocellular ballooning was scored as grade 0 for none, grade 1 for few ballooned cells, and grade 2 for predominant ballooning. Hepatic inflammatory infiltrates were scored as grade 0 for none, grade 1 for <2 foci/field, grade 2 for 2-4 foci/field, or grade 3 for >4 foci/field.

3.3.5. Lipids and Clinical Assays

Hepatic total lipid was determined gravimetrically [14], and solubilized to determine triglyceride, cholesterol (Pointe Scientific, Canton, MI) and FFA (Wako Diagnostics, Mountain View, CA) using spectrophotometric kits. Serum lipids were determined directly with these kits. Serum glucose was measured by clinical assay (Pointe Scientific) and insulin by ELISA (Crystal Chem, Downers Grove, IL). Serum ALT activity was measured by clinical assay (Pointe Scientific).

3.3.6. RT-PCR

Gene expression studies were performed as described [15], with minor modifications. In brief, total RNA was extracted using TRIzol (Invitrogen). RT-PCR was performed using a SYBR Green PCR Kit, a Bio-Rad CFX384 instrument (Hercules, CA), and primer sequences purchased from Sigma (Table 1). mRNA expression of human Nrf2 in HC-04 cells were quantified relative to β-actin using the \(2^{-\Delta\Delta CT}\) method [200]. For studies in mice, hepatic expression of genes involved in antioxidant defense (Nrf2; Nqo1), lipid metabolism [cluster of differentiation-36 (CD36); SREBP-1c; FAS; stearoyl-CoA desaturase-1 (SCD1); diglyceride acyltransferase (DGAT1/2)] and inflammation [MCP-1, TNFα, iNOS; TLR4; TNFR1] were quantified relative to hypoxanthine-guanine phosphoribosyl transferase (HPRT).
3.3.7. Western Blotting

Total cellular proteins were extracted from liver using a Pierce T-PER kit containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Proteins were denatured under reducing conditions, separated on a 10% SDS-PAGE, and transferred to a nitrocellulose membrane. For studies in HC-04 cells, membranes were probed overnight with primary antibodies against Nrf2 (#12721; Cell Signaling Technology; Danvers, MA) or TFIIB (#SC-225; Santa Cruz Biotechnology; Dallas, Texas) that were diluted 1:2000 in 2% non-fat dried milk in TBST. For studies in mice, membranes were probed for 1 h at room temperature with primary antibody against β-actin (#4967; Cell Signaling Technology) that was diluted 1:1000 in TBST, or they were probed overnight at 4°C with antibodies against phosphorylated-p65 (phospho-p65; 1:500; #3033) and p65 (1:1000; #8242) prepared in TBST containing 5% (w/v) BSA. After washing in TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:1000; #SC-2056; Santa Cruz Biotechnology). Detection was accomplished by enhanced chemiluminescence and densitometry analysis on a Kodak 2000RT imaging station using 1D software.

3.3.8. Hepatic Antioxidants and Lipid Peroxidation

Hepatic ascorbic acid and uric acid were measured by HPLC as described [14], with minor modification to use an UltiMate 3000 HPLC system (Thermo Scientific) equipped with two 6011RS coulometric cells programmed to 25, 150, 250, and 350 mV. Hepatic α-tocopherol was measured by LC-MS as we described [201] following saponification and hexane extraction. Hepatic MDA was extracted and measured by HPLC-FL as described [15], with minor modifications. In brief, liver was homogenized in lysis buffer (Gold Biotechnology; St. Louis, MO) containing 5 mM DL-dithiothreitol
(Sigma), 5 mM EDTA disodium salt, and protease and phosphatase inhibitor cocktail (Thermo Scientific). Following centrifugation, the supernatant was mixed with TBA, incubated, and the butanol extract was injected on a HPLC-FL system set to 532/553 nm (excitation/emission). Separation was performed at 1 mL/min on an Alltima C18 column (250 x 4.6 mm, 5 μm; Columbia, MD) using 50:50 methanol and phosphate buffer (pH 6.5). MDA was quantified from standards prepared in parallel from TPP, and normalized to hepatic protein.

3.3.9. Statistical Analysis

Data (means ± SEM) were analyzed using GraphPad Prism, version 6 (GraphPad Software; La Jolla, CA). 1-way or 2-way ANOVA was used as appropriate for studies performed in HC-04 cells. For mouse studies, 2-way ANOVA was used to evaluate main effects due to genotype and GTE, and their interaction. Variables with unequal variance were log-transformed to achieve a normal distribution. Newman-Keuls post-hoc test was used to evaluate group mean differences following statistically significant main or interactive effects as appropriate. Groups without a common superscript were statistically significant different at P≤0.05.

3.4. Results

3.4.1. EGCG increases nuclear Nrf2 accumulation in vitro and dietary GTE increases hepatic Nrf2 expression

Studies in HC-04 cells were conducted to define the effect of physiologic concentrations of EGCG on nuclear accumulation of Nrf2. Western blotting studies indicated that 2 h treatment with EGCG (0.1-5 μM) increased nuclear accumulation of Nrf2 at all concentrations tested (Figure 3A). This occurred without any time (0-2 h)- or concentration (0-5 μM)-dependent changes in Nrf2 mRNA expression (Figure 3B).
Increases in nuclear Nrf2 accumulation by EGCG also occurred without affecting cytotoxicity (Figure 3C-D). Further corroborating, livers of mice chronically fed an HF diet containing GTE showed increased (P<0.05) mRNA expression of Nrf2 and its transcriptional target Nqo1 compared to mice fed an HF diet containing no GTE (Figure 4). Expression of these genes in Nrf2-null mice were lower than WT mice regardless of GTE. Collectively, data support that hepatoprotective activities of green tea observed in rodent models of NASH [9] may be mediated through Nrf2.

3.4.2. GTE reduces body mass independent of food intake and Nrf2 status, but reductions in adiposity by GTE are partly Nrf2-dependent

At study onset, body mass was not different between Nrf2-null and WT mice (Table 2). Following the 8 wk feeding period, body mass of Nrf2-null mice was lower than those of WT mice despite no significant difference in food intake (P>0.05). Body masses of WT and Nrf2-null mice fed GTE were lower than those of their respective controls, but without any genotype differences. Liver and adipose mass did not differ between Nrf2-null and WT mice. However, GTE lowered liver mass to a similar extent regardless of genotype, whereas adiposity was lowered by GTE to a greater extent in WT mice compared to Nrf2-null mice (Table 2).

3.4.3. GTE reduces hepatic steatosis and injury regardless of Nrf2 deficiency

Histologic evaluation provided clear visual evidence that Nrf2-null mice had exacerbated liver steatosis and hepatocellular ballooning compared to WT mice (Figure 5). Although GTE reduced these parameters in both genotypes, their reduction was more pronounced in WT mice fed GTE in which there was little or no histologic evidence of steatosis or ballooning. In contrast, inflammatory infiltration was minimal regardless of genotype or GTE.
Hepatic lipid was quantified to corroborate histologic evidence of steatosis (Table 2). A genotype x GTE interaction ($P<0.05$) indicated that hepatic total lipid was increased in Nrf2-null mice compared with WT mice, but that GTE reduced it to lower levels in WT mice compared to Nrf2-null mice. Higher hepatic total lipid in Nrf2-null mice was attributed to greater hepatic triglyceride and cholesterol (Table 2). However, hepatic cholesterol within each genotype was unaffected by GTE, whereas hepatic triglyceride was lowered by GTE regardless of genotype, and more greatly lowered in WT mice fed GTE. Hepatic FFA was unaffected by Nrf2 or GTE.

Serum ALT activity, a biomarker of liver injury, was greater in Nrf2-null mice compared to WT mice (Table 2). Serum ALT was lower in WT and Nrf2-null mice fed GTE compared to their respective controls with no difference in ALT between WT and Nrf2-null mice fed GTE. Serum glucose was unaffected by genotype, but was reduced to a similar extent by GTE in both WT and Nrf2-null mice (Table 2). Serum insulin was higher in Nrf2-null mice compared to WT mice. GTE lowered insulin levels in both genotypes relative to respective controls with no differences between WT and Nrf2-null mice fed GTE. Lastly, serum FFA was unaffected in Nrf2-null mice, but was lowered by GTE in WT mice only (Table 2). Together, an HF diet exacerbates liver steatosis and injury, and insulinemia in Nrf2-null mice, but GTE-mediated improvements in these parameters occurred largely independent of Nrf2 status.

3.4.4. GTE lowers mRNA expression of hepatic lipid uptake and lipogenic genes regardless of Nrf2 status

To assess hepatic lipid-lowering activities of GTE, expression of genes involved in FFA uptake and lipogenesis were examined (Figure 6). Nrf2-null mice compared with WT mice had increased mRNA of the fatty acid uptake transporter CD36 as well as the
lipogenic transcription factor SREBP-1c and its downstream targets FAS and SCD1. Nrf2-null mice also had greater DGAT-1 and -2 mRNA compared with WT mice. Although GTE reduced expression of each gene, GTE lowered their expression to a greater extent in WT mice fed GTE compared to Nrf2-null mice fed GTE with the exception of DGAT-1 and -2, which were reduced to a similar extent by GTE in both genotypes. Thus, liver steatosis in Nrf2-null mice is likely exacerbated by greater hepatic uptake of FFA and lipid synthesis, but GTE likely only provides limited Nrf2-dependent hepatoprotection on these parameters.

3.4.5. GTE lowers lipid peroxidation otherwise increased by Nrf2 deficiency without improving antioxidant status

Consistent with greater liver steatosis (Figure 5), Nrf2-null mice had greater hepatic MDA compared to WT controls (Figure 7A). Although MDA was significantly lower in both WT and Nrf2-null mice fed GTE compared to their respective controls, MDA in Nrf2-null mice fed GTE remained significantly higher than those of WT mice fed no GTE (Figure 7A). Endogenous and dietary antioxidants known to regulate oxidative stress were then assessed to better define the interaction of GTE and Nrf2 status on hepatic lipid peroxidation. Ascorbic acid, which is devoid from the purified diet because rodents synthesize it, was lower in Nrf2-null mice and unaffected by GTE regardless of genotype (Figure 7B). Uric acid, a byproduct of purine catabolism that has antioxidant activity at physiologic levels [202], was lower in Nrf2-null mice and unaffected by GTE in both WT and Nrf2-null mice (Figure 7C). To the contrary, Nrf2-null mice had greater accumulation of hepatic α-tocopherol compared to WT mice (Figure 7D). Nrf2-null mice fed GTE had α-tocopherol levels lowered to the extent of WT controls fed no GTE, whereas α-tocopherol of WT mice fed GTE was lower than
that of WT mice. However, normalization of α-tocopherol to total hepatic lipid resulted in Nrf2-null mice having greater α-tocopherol, but without any effect of GTE in either genotype (data not shown). Collectively, Nrf2 deficiency exacerbates hepatic MDA, but GTE-mediated improvements in lipid peroxidation occur without improving hepatic antioxidants.

3.4.6. GTE reduces inflammatory responses otherwise increased by Nrf2 deficiency

NFκB activation increases with oxidative stress and contributes to hepatic injury during NASH [16]. Consistent with GTE reducing NFκB-dependent inflammatory responses during NASH in association with lowered oxidative stress [15, 16], we hypothesized that anti-inflammatory activities of GTE would occur in an Nrf2-dependent manner. Consistent with our hypothesis, Nrf2-null mice had greater protein levels of phospho-p65, a transcriptionally active form of NFκB [203], compared with WT mice (Figure 8). Phospho-p65 levels were lowered in WT mice fed GTE. However, and contrary to our hypothesis, GTE also lowered phospho-p65 levels in Nrf2-null mice to the extent observed in WT mice fed GTE. These decreases in phospho-p65 occurred without any Nrf2- or GTE-mediated alterations in total p65 protein (Figure 8).

To corroborate the opposing activities of Nrf2 status and GTE on NFκB activation, expression of NFκB-dependent target genes implicated in NASH were assessed. Consistent with Nrf2-null mice having increased phospho-p65 protein, they also had greater TNFα, MCP-1, and iNOS mRNA compared with WT mice (Figure 9). GTE lowered mRNA levels of each of these pro-inflammatory targets regardless of genotype. However, iNOS mRNA in Nrf2-null mice fed GTE was only lowered to the extent observed in WT mice, whereas TNFα and MCP-1 were lowered to levels not
different from WT mice fed GTE. Thus, while Nrf2 deficiency increases NFκB-dependent inflammation, anti-inflammatory activities of GTE during NASH appear to be mediated largely in an Nrf2-independent manner. Consistent with this notion, we considered an alternative hypothesis that anti-inflammatory activities of GTE could be mediated by cellular receptors that trigger NFκB activation during NASH, specifically TNFR1 and TLR4 [115, 167]. Indeed, hepatic mRNA expression of both TNFR1 and TLR4 was higher in Nrf2-null mice compared with WT mice (Figure 10). GTE reduced TNFR1 and TLR4 mRNA expression regardless of genotype with no difference in mRNA levels between genotypes.

3.5. Discussion

Consistent with our hypothesis, and the work of others [156], Nrf2 deficient mice fed an HF diet had exacerbated NASH pathology as evidenced by greater NFκB-dependent pro-inflammatory responses, along with increased hepatic lipid peroxidation, liver steatosis, and expression of hepatic FFA uptake and lipogenic genes. Consistent with evidence suggesting that Nrf2-mediated hepatoprotective activities of GTE during NASH [15, 16] are likely mediated through EGCG [9], our data support that GTE and EGCG can increases the transcriptional activity of Nrf2. Contrary to our hypothesis, our data suggest that GTE provides limited Nrf2-dependent protection against liver steatosis and injury, lipogenic gene expression, and activation of NFκB and expression of its pro-inflammatory mediators. In addition, although GTE lowers hepatic lipid peroxidation regardless of Nrf2 status, this occurred without any GTE-mediated improvements in hepatic antioxidants in Nrf2-null mice. Collectively, anti-inflammatory and hypolipidemic activities of GTE during NASH likely occur largely through a mechanism independent of Nrf2 activation.
High dietary green tea intakes (≥10 servings/d) are associated with lower biomarker levels of liver injury, inflammation, and hyperlipidemia [139]. In agreement, evidence in genetic and diet-induced rodent models of NASH support that dietary GTE or its principal catechin EGCG reduces liver steatosis and injury, insulin resistance, oxidative stress, and inflammation [9]. Although the mechanism underlying these health benefits remains unclear, hepatoprotective mechanisms of GTE likely occur through both Nrf2-independent and -dependent mechanisms. For example, EGCG reduces intestinal lipid absorption by inhibiting pancreatic phospholipase A\textsubscript{2} activity [141] and may mitigate insulin resistance by reducing postprandial glucose resulting from starch ingestion by inhibiting α-amylase activity [204]. In contrast, studies in ob/ob mice and rats fed an HF diet support an Nrf2-dependent protective mechanism consistent with GTE upregulating enzymatic activities of antioxidant defenses under the transcriptional control of Nrf2 (e.g. glutathione S-transferase, glutathione peroxidase, catalase) [15]. GTE also reduced NFκB activation and expression of TNFα and MCP-1 that were otherwise increased by an HF diet, and their lowering occurred in association with improved glutathione status [16], consistent with studies in ob/ob mice showing that GTE increases the expression of Nrf2-dependent genes regulating glutathione biosynthesis [15].

Supporting our hypothesis that GTE would protect against NASH in an Nrf2-dependent manner, we show that physiologically relevant concentrations (0.1-5 μM) of EGCG [9], a prominent constituent of GTE, increase nuclear protein accumulation of Nrf2 in HC-04 cells in agreement with studies in vitro and in rodent models indicating EGCG-mediated activation of Nrf2-dependent targets that detoxify ROS [160]. This cytoprotective response is thought to be mediated through the electrophilic potential of
EGCG [160], which is readily auto-oxidized at near-neutral pH similar to that occurring during digestion [161]. The EGCG electrophile is then expected to promote oxidative modification of cysteine residues on Keap1. This induces conformational changes of the Keap1-Nrf2 complex to suppress Nrf2 degradation by limiting ubiquitination, and facilitates nuclear translocation of newly synthesized Nrf2 where it binds to antioxidant response elements to regulate the expression of antioxidant and xenobiotic defenses [158, 159]. Consistent with these findings, we show that GTE in WT mice fed an HF diet, but not in Nrf2-null mice, increases mRNA expression of Nrf2 and its downstream target gene Nqo1, which assists in lowering oxidative stress by maintaining cellular antioxidants in their reduced form, scavenging superoxide, and catalyzing reductive metabolism of xenobiotics [156]. Although numerous antioxidant defenses largely regulated by Nrf2 are also regulated by other transcription factors [205], the present study aimed to define the extent to which antioxidant activities of GTE protect against NASH in an Nrf2-dependent manner by lowering NFκB-dependent inflammation and hepatic lipid accumulation, which have been shown to be attenuated by dietary GTE or EGCG in obese models of NASH [14-16, 45, 144].

NFκB can be activated in a redox-dependent manner by oxidative stress. In agreement, Nrf2-null mice fed a MCD diet to induce NASH have greater lipid peroxidation along with nuclear accumulation of the p65 subunit of NFκB and mRNA expression of its pro-inflammatory mediators [154]. In our study, livers of Nrf2-null mice fed an HF diet have increased phospho-p65 protein and TNFα, MCP-1, and iNOS mRNA expression along with higher MDA compared to WT controls. GTE lowered hepatic NFκB binding activity in rats fed an HF diet [16]. This is corroborated in our study by GTE-mediated decreases in phospho-p65 protein accumulation and TNFα
and MCP-1 mRNA expression in WT mice, consistent with EGCG reducing circulating MCP-1 in mice fed an HF diet [198]. However, these inflammatory responses in Nrf2-null mice fed GTE were also lowered, and to levels no different from WT mice fed GTE, suggesting that anti-inflammatory activities of GTE occur in an Nrf2-independent manner. Furthermore, the hepatic antioxidants ascorbic acid and uric acid, a byproduct of purine metabolism known to be regulated by Nrf2 [206], were also lowered by Nrf2 deficiency, and otherwise unaffected by GTE regardless of Nrf2 status.

Insulin resistance is an early event leading to NASH [188], although direct evidence also shows that TNFα-mediated inflammation accelerates the induction of liver steatosis in mice [81]. In agreement with others [207], and the present evidence that Nrf2 deficiency increases NFκB-mediated inflammation, we also show that Nrf2-null mice have exacerbated histologic and biochemical markers of liver steatosis compared to WT mice. Greater liver steatosis in Nrf2-null mice is consistent with a mechanism of enhanced fatty acid uptake by CD36 and greater lipid synthesis mediated by SREBP1c and its downstream lipogenic genes FAS and SCD1 that are upregulated in association with hyperinsulinemia. However, these Nrf2-dependent activities that likely promote hepatic steatosis occurred without affecting hepatic FFA, which may reflect their rapid esterification to form triglyceride that is mediated by the observed increase in DGAT expression and needed to protect against FFA-induced hepatic lipotoxicity [48].

Consistent with our studies showing GTE-mediated improvements in liver steatosis in association with lowered serum insulin and expression of lipogenic genes [15, 144], the present study shows that GTE reduced liver steatosis regardless of Nrf2 deficiency. Although biochemical measures of hepatic total lipid and triglyceride in Nrf2-
null mice fed GTE were lowered compared to Nrf2-null controls, they were not lowered to the extent observed in WT mice fed GTE. This suggests that a small proportion of the hypolipidemic activities of GTE occur in an Nrf2-dependent manner. The observed lipid-lowering activity is corroborated by our findings that GTE in Nrf2-null mice lowered the expression of CD36 and lipogenic genes (i.e. SREBP1c, FAS, SCD1) only to the extent observed in WT controls, but not WT mice fed GTE. Although this study was not designed to define events downstream of Nrf2 activation responsible for these hypolipidemic activities, nor could it isolate the anti-inflammatory activities of GTE from its hepatic lipid-lowering activities, Nrf2 maintains energy metabolism by regulating genes for lipid biosynthesis and mitochondrial biogenesis [208] and warrants further investigation. Likewise, consideration for examining Nrf1-dependent hepatoprotection is warranted consistent with Nrf1 transcriptional activities similarly mediated through its binding to ARE to regulate lipid metabolism [147]. However, Nrf1 limits steady-state cellular stress whereas Nrf2 provides secondary defense against unscheduled stress [147], and may have a role in mitigating NASH. Indeed, studies in liver-specific Nrf1 knockout mice show that Nrf1 regulates hepatic lipid metabolism [209] consistent with Nrf1 regulating the expression of PPARα and PPARγ coactivators [210]. Thus, GTE may attenuate NASH through Nrf1 consistent with EGCG reducing liver steatosis in HF-fed mice while upregulating skeletal muscle mRNA expression of Nrf1 and fatty acid oxidation genes [211].

Inflammation contributes to NASH consistent with NASH patients and rodents models of NASH having greater NFκB activation [16, 75, 76]. Although our findings implicate Nrf2 deficiency in increasing NFκB-dependent inflammation, they also suggest that anti-inflammatory activities of GTE on NFκB activation occur independent
of Nrf2 responses that suppress ROS. Indeed, HF feeding is known to induce Nrf2 cytoprotective responses that limit ROS accumulation [212]. Thus, GTE may be limited in further upregulating Nrf2-dependent defenses, and supports examining Nrf2-dependent protection by GTE in other NASH models, consistent with the flavone baicalein, an Nrf2 activator, suppressing liver steatosis and inflammation in mice fed a MCD diet [213].

Mechanisms other than Nrf2-mediated alterations in intracellular redox status, such as receptor-mediated pathways, are also known to regulate NFκB activation during NASH [20]. Greater inflammation in NASH patients is associated with small intestinal bacteria overgrowth and greater TLR4 expression on CD14 positive cells that is correlated to plasma IL-8 [115]. Alternatively, studies in TNFR1 knockout mice [167], TNFR1 knockin mice that are unable to down-regulate TNFα-dependent inflammation [168], or treatment with anti-TNFα antibodies [85] support that inhibiting TNFR signaling attenuates NASH. Consistent with these lines of study, we show that hepatic TNFR1 and TLR4 expression that are otherwise increased by Nrf2 deficiency are normalized by GTE in Nrf2-null mice to the extent observed in WT mice fed GTE. Thus, these receptor-mediated pathways warrant consideration to better define anti-inflammatory activities of GTE associated with lowered hepatic TNFα by GTE in obese models of NASH [15, 16] as well as studies showing that EGCG suppresses TLR4 signaling in LPS-stimulated adipocytes [214] and reduces hepatic TLR4 protein in a mouse model of viral hepatitis [215]. Evidence also supports that polyphenols favorably improve the intestinal microbiota composition to mitigate inflammatory-related disorders [120].

In conclusion, GTE protects against HF-induced liver injury and NASH by lowering hepatic lipid accumulation and NFκB-dependent inflammation. Suppression of NFκB-
dependent inflammation by GTE is likely independent of Nrf2 signaling and these anti-inflammatory activities may contribute, at least in part, to its hepatic lipid-lowering activities. Identifying the mechanism by which GTE mitigates NFκB-dependent inflammation, such as that mediated through TNFR1 and/or TLR4, warrants further investigation. Furthermore, it is likely that pleiotropic effects of GTE may protect against NASH through multiple mechanisms, consistent with EGCG regulating lipid metabolism by activating PPARα and inhibiting PPARγ [144]. Regardless of the mechanism, green tea is among the most commonly used dietary supplements, it is considered safe when ingested as recommended [216], and it or EGCG may provide therapeutic potential in protecting obese individuals from NASH.
Figure 3. Time- and dose-response effects of EGCG on cell cytotoxicity and nuclear accumulation and mRNA expression of Nrf2 in HC-04 human hepatocytes.

HC-04 were treated with 0-5 µM EGCG for up to 2 h as described under Methods. A) Representative Western blot of Nrf2 from isolated nuclear fractions of HC-04 cells following 2 h incubation with EGCG or 100 µM t-butylhydroperoxide (positive control). B) HC-04 cells were treated for up to 2 h with 0.1-5 µM EGCG before isolating total RNA using Trizol and reverse transcribing for RT-PCR analysis of Nrf2 mRNA expression. C) LDH leakage and D) cell viability were assessed using spectrophotometric kits as described under Methods following treatment of HC-04 for 2 h with 0-5 µM EGCG. Shown are mean responses of experiments performed in triplicate. Data were analyzed by 1- or 2-way ANOVA as appropriate. There were no statistically significant time- or concentration-dependent effects of EGCG. Abbreviations: EGCG, epigallocatechin gallate; LDH, lactate dehydrogenase; Nrf2, nuclear factor erythroid-2-related factor-2.
Figure 4. Hepatic mRNA expression of Nrf2 and Nqo1 in Nrf2-null and wild-type mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

A) Nrf2. B) Nqo1. RNA was isolated using Trizol and reverse transcribed for RT-PCR analysis using the primers described in Table 1. Data (means ± SEM, n = 10-12 mice per group) were analyzed by 2-way ANOVA with Newman–Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different (P<0.05). Abbreviations: GTE, green tea extract; Nrf2, nuclear factor erythroid-2-related factor-2; Nqo1, NADPH:quinone oxidoreductase 1; WT, wild-type.
Figure 5. Histologic evaluation of livers from Nrf2-null and wild-type mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

A) Representative hematoxylin and eosin-stained liver sections (original magnification 200x) in WT and Nrf2-null mice fed a high fat with or without GTE. Nrf2-null mice had exacerbated macrovesicular steatosis and hepatocyte ballooning relative to WT mice. GTE reduced these parameters regardless of genotype, but the effects were more pronounced in WT mice. Histologic scoring of liver steatosis (B), hepatocyte ballooning (C), and inflammatory infiltrates. Data were analyzed by 2-way ANOVA with Newman–Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different (P<0.05). Data are means ± SEM, n = 10-12 mice per group. Abbreviations: GTE, green tea extract; Nrf2, nuclear factor erythroid-2-related factor-2; WT, wild-type.
Figure 6. Hepatic mRNA expression of genes involved in FFA uptake and lipid synthesis in Nrf2-null and wild-type mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

RNA was isolated using Trizol and reverse transcribed for RT-PCR analysis using the primers described in Table 1. Data (means ± SEM, n = 10-12 mice per group) were analyzed by 2-way ANOVA with Newman–Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different (P<0.05). Abbreviations: CD36, cluster of differentiation-36; DGAT, diglyceride acyltransferase; FAS, fatty acid synthase; FFA, free fatty acids; GTE, green tea extract; Nrf2, nuclear factor erythroid-2-related factor-2; SCD1, stearoyl-CoA desaturase-1; SREBP-1c, sterol regulatory element binding protein-1c; WT, wild-type.
Figure 7. Hepatic lipid peroxidation and antioxidant markers in Nrf2-null and wild-type mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

A) Hepatic MDA was measured by HPLC-FL following incubation of liver homogenates with thiobarbituric acid and extraction with butanol. B-C) Hepatic ascorbic acid and uric acid were measured by HPLC-ECD following deproteinization of liver homogenates. D) Hepatic α-tocopherol was measured by HPLC-ECD following alcoholic saponification and extraction with hexane. Data (means ± SEM, n = 10-12 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different (P<0.05). Abbreviations: GTE, green tea extract; MDA, malondialdehyde; Nrf2, nuclear factor erythroid-2-related factor-2; WT, wild-type.
Figure 8. Hepatic protein of the phosphorylated p65 subunit of NFκB or total p65 in Nrf2-null and wild-type mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

A) Representative Western blot of phospho-p65 and p65 and the loading control β-actin. B) Quantitative densitometry analysis of target protein accumulation in total cellular extracts. Data (means ± SEM, n = 10-12 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different (P<0.05). Abbreviations: GTE, green tea extract; Nrf2, nuclear factor erythroid-2-related factor-2; phospho-p65; phosphorylated p65 subunit of NFκB; WT, wild-type.
Figure 9. Hepatic mRNA expression of pro-inflammatory genes in Nrf2-null and wild-type mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

RNA was isolated using Trizol and reverse transcribed for RT-PCR analysis for the NFκB-dependent targets TNFα, MCP-1, and iNOS using the primers described in Table 1. Data (means ± SEM, n = 10-12 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different (P<0.05). Abbreviations: GTE, green tea extract; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein-1; Nrf2, nuclear factor erythroid-2-related factor-2; TNFα, tumor necrosis factor-α; phospho-p65; WT, wild-type.
Figure 10. Hepatic mRNA expression of pro-inflammatory genes in Nrf2-null and wild-type mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

RNA was isolated using Trizol and reverse transcribed for RT-PCR analysis for the NFκB-dependent targets TNFα, MCP-1, and iNOS using the primers described in Table 1. Data (means ± SEM, n = 10-12 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different (P<0.05). Abbreviations: GTE, green tea extract; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein-1; Nrf2, nuclear factor erythroid-2-related factor-2; TNFα, tumor necrosis factor-α: phospho-p65; WT, wild-type.
Table 1. Primers used for RT-PCR gene expression studies in Chapter 3

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin*</td>
<td>CCATCGAGCACGGGCATC</td>
<td>ATTGTAGAAGGTGTGGTGAGCA</td>
</tr>
<tr>
<td>CD36</td>
<td>CCGAGGACCACACTGTGC</td>
<td>AACCACCAAAGAGTTCTTTCAA</td>
</tr>
<tr>
<td>DGAT1</td>
<td>TCCGCTCTGGGCATTC</td>
<td>GAATCGGGCCACAAATCCA</td>
</tr>
<tr>
<td>DGAT2</td>
<td>AGTGGCAATGCTATCATCGT</td>
<td>AAGGAATAAGTGGAACCAGATCA</td>
</tr>
<tr>
<td>FAS</td>
<td>GGAGGTGTTGATAGCCGTTAT</td>
<td>TGGGTAATCCATAGAGCCAG</td>
</tr>
<tr>
<td>HPRT</td>
<td>CACAGGACTAGAACACCTGC</td>
<td>GCTGGTGAAAGGACCTCT</td>
</tr>
<tr>
<td>iNOS</td>
<td>TTCTGTGCTGTCCAGTGAG</td>
<td>TGAAGAAAACCCCTTGTGCT</td>
</tr>
<tr>
<td>MCP-1</td>
<td>TGATCCCAATGAGTAGCTGGAG</td>
<td>ATGTCTGGACCATTCTCTTCTT</td>
</tr>
<tr>
<td>Nrf2*</td>
<td>GCTCATACTCTTTCGTCGC</td>
<td>ATCATGATGGACTTGGAGGTG</td>
</tr>
<tr>
<td>Nrf2</td>
<td>CGAGATATACGCAGGAGAGGTAAGA</td>
<td>GTCGAACAATGGTTCTCCAGTT</td>
</tr>
<tr>
<td>Nqo1</td>
<td>TTCTGTGGCTCCAGGTCTT</td>
<td>AGGCTGGTTGGAGCAAATA</td>
</tr>
<tr>
<td>SCD1</td>
<td>TTCTTGGCATACACTCTGGTGC</td>
<td>CCGGATTGAATTTCTCTGCTG</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>GCAGCCACCACCTAGCTTG</td>
<td>CAGCAGTGAGTCTGCTTGGAT</td>
</tr>
<tr>
<td>TLR4</td>
<td>CCTCTGCTCTACACTAGAGACTT</td>
<td>TGTGGAGCCCTCTCCTGGAT</td>
</tr>
<tr>
<td>TNFα</td>
<td>CTCCAGGCGGTGCCTATG</td>
<td>GGGCCATAGAAGTGTAGAGG</td>
</tr>
</tbody>
</table>

Continued
| TNFR1 | GGGCACCTTTACGGCTTCC | GGTTCTCTTTACAGCCACACA |

All primer sequences are for mice except for human sequences that are annotated by an asterisk. Abbreviations: CD36, cluster of differentiation-36; DGAT, diglyceride acyltransferase; FAS, fatty acid synthase; HPRT, hypoxanthine-guanine phosphoribosyl transferase; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein-1; Nrf2, nuclear factor erythroid-2-related factor-2; Nqo1, NAD(P)H dehydrogenase:quinone 1; SCD1, stearoyl-CoA desaturase-1; SREBP-1c, sterol regulatory element binding protein-1c; TLR4, toll-like receptor-4; TNFα, tumor necrosis factor-α; TNFR1, tumor necrosis factor receptor-1
Table 2. Body composition, food intake, and serum and liver metabolic parameters in Nrf2-null and wild-type mice fed an HF diet containing GTE at 0% or 2% for 8 wk.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>WT + GTE</th>
<th>Nrf2-null</th>
<th>Nrf2-null + GTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Body Weight (g)</td>
<td>26.9 ± 0.4</td>
<td>27.0 ± 0.5</td>
<td>28.4 ± 0.8</td>
<td>28.9 ± 0.6</td>
</tr>
<tr>
<td>Final Body Weight (g)</td>
<td>29.5 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.8 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.0 ± 1.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>Food Intake (g/d)</td>
<td>2.9 ± 0.1</td>
<td>2.5 ± 0.3</td>
<td>2.3 ± 0.1</td>
<td>0.87 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver Mass (g)</td>
<td>1.40 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.05 ± 0.0&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.16 ± 0.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.87 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Adipose Tissue (g)</td>
<td>3.93 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.03 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.34 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.12 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hepatic Total Lipid (mg/g liver)</td>
<td>231.7 ± 16.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>156.9 ± 13.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>312.5 ± 19.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>219.2 ± 17.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hepatic Triglyceride (μmol/g liver)</td>
<td>34.4 ± 4.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>122.7 ± 13.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.1 ± 10.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>71.1 ± 10.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hepatic Cholesterol (μmol/g liver)</td>
<td>8.4 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.3 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.9 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.9 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hepatic FFA (μmol/g liver)</td>
<td>35.4 ± 5.4</td>
<td>31.5 ± 3.9</td>
<td>34.4 ± 2.2</td>
<td>34.4 ± 2.2</td>
</tr>
<tr>
<td>Serum ALT (U/L)</td>
<td>28.5 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.0 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>85.8 ± 13.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.5 ± 6.9&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum Glucose (mmol/L)</td>
<td>17.6 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.0 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.7 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.4 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum Insulin (pmol/L)</td>
<td>90.2 ± 8.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52.9 ± 7.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>123.7 ± 9.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63.8 ± 11.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum FFA (mmol/L)</td>
<td>0.94 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.68 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.93 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.93 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Total adipose mass represents the sum of epididymal and retroperitoneal fat pads. Data are means ± SEM, n = 10-12 in each group. Data were analyzed by 2-way ANOVA with Newman-Keuls post-test to assess group mean differences. Means in a row not sharing a common letter are significantly different, *P* ≤ 0.05. Abbreviations: ALT, alanine aminotransferase; FFA, free fatty acids; GTE, green tea extract; HF, high-fat; Nrf2, nuclear factor erythroid-2-related factor-2; WT, wild-type.
CHAPTER 4
GREEN TEA EXTRACT TREATMENT REDUCES NFκB ACTIVATION IN MICE WITH DIET-INDUCED NONALCOHOLIC STEATOHEPATITIS BY LOWERING TNFR1 AND TLR4 EXPRESSION AND LIGAND AVAILABILITY

4.1. Abstract

NFκB-mediated inflammation contributes to liver injury during NASH. We hypothesized that antiinflammatory activities of GTE during NASH would lower tumor necrosis factor TNFR1- and TLR4-mediated NFκB activation. Male C57BL/6J mice (6 wk old) were fed an LF or HF diet for 12 wk to induce NASH. They were then randomized to continue on these diets supplemented with 0 or 2% GTE (n = 10/group) for an additional 8 wk prior to evaluating NASH, NFκB inflammation, and TNFR1 and TLR4 receptor complexes and their respective ligands TNFα and endotoxin. HF feeding increased (P<0.05) serum ALT activity and histological evidence of NASH compared with LF controls. HF-mediated increases in NFκB p65 phosphorylation were also accompanied by increased serum TNFα and endotoxin concentrations, and mRNA expression of hepatic TNFR1 and TLR4, and MyD88 protein levels. GTE in LF mice had no effect (P>0.05) on liver histology or inflammatory responses. However, GTE in HF mice decreased biochemical and histological parameters of NASH and lowered hepatic p65 phosphorylation in association with decreased serum TNFα, mRNA expression of TNFR1 and TLR4, and MyD88 protein. GTE in HF-fed mice also lowered
serum endotoxin and upregulated mRNA expression of duodenal occludin and zonula occluden-1, and ileal occludin and claudin-1 that were otherwise lowered in expression by HF-feeding. These data suggest that dietary GTE treatment reduces hepatic inflammation in NASH by decreasing pro-inflammatory signaling through TNFR1 and TLR4 that otherwise increases NFκB activation and liver injury.

4.2. Introduction

NAFLD is the term used to describe several liver diseases that advance in severity from relatively benign steatosis, to NASH, fibrosis, and cirrhosis, and collectively increase the risk for hepatocellular carcinoma and liver failure [1]. NAFLD is the most common liver disease in the United States [27] with 80-100 million Americans afflicted by this potentially life-threatening progressive disorder [2]. Of significant concern is that the already high prevalence of NAFLD is expected to worsen due to its close relationship with obesity and the lack of established therapies beyond weight management [217].

The progression to NASH is often described by a “two-hit” mechanism [188] in which a “first-hit” mediated by insulin resistance promotes excess hepatic triglyceride accumulation to result in steatosis. Steatotic livers are then vulnerable to reactive oxygen species (ROS)-mediated “second-hits” that increase hepatic NFκB activation and expression of pro-inflammatory proteins leading to liver injury [188]. Increased NFκB activation occurs in both patients [75] and in rodent models of NASH [218]. NFκB activation is mechanistically linked to NASH due to its role in impairing hepatic VLDL export, increasing mitochondrial dysfunction, and enhancing the hydrolysis and flux of adipose-derived fatty acids to the liver [54, 87, 89]. Thus, therapies that inhibit NFκB-mediated inflammation would be expected to effectively manage NASH.
NFκB is activated following the phosphorylation of IκB, which triggers the translocation of NFκB to the nucleus where it transcriptionally upregulates the expression of pro-inflammatory genes (e.g. TNFα) [195]. Prominent targets to regulate NFκB activation during NASH include those mediated intracellularly from ROS and extracellularly from signaling mediated by TLR4 and TNFR1 [219]. Indeed, hepatic NFκB binding activity that is increased in a rodent model of dietary fat-induced NASH is also inversely correlated with hepatic glutathione [16]. This supports that increased intracellular ROS promotes NFκB-mediated inflammation. Approaches that attenuate TNFR1 signaling may also protect against NASH. For example, NASH patients have increased expression of TNFR1, but not TNFR2 [169], and obese mice treated with TNF antibody have lower NFκB binding activity [85]. Loss-of-function TLR4 mutant mice are also protected from diet-induced NASH consistent with a lowering of NFκB-mediated inflammatory responses despite high portal vein concentrations of endotoxin (i.e. LPS), a TLR4 ligand [96].

We and others have demonstrated that GTE, or its major catechin EGCG, functions to protect against obesity-mediated NASH through antioxidant and antiinflammatory mechanisms [9]. In a rodent model of HF-induced NASH, dietary GTE attenuated hepatic NFκB binding activity and expression of TNFα and monocyte chemoattractant protein-1 [16]. These antiinflammatory activities occurred in association with increased concentrations of hepatic glutathione, an antioxidant that is synthesized in an Nrf2-dependent manner [16]. However, our study in Nrf2-knockout (KO) mice demonstrates that GTE limits NFκB activation during NASH in an Nrf2-independent manner [25], suggesting that the antiinflammatory activities of GTE are unlikely to be attributed to an Nrf2-dependent antioxidant function. In the same study
[25], GTE attenuated mRNA expression of TNFR1 and TLR4 in both wild-type and Nrf2-KO mice. This supports the possibility that GTE attenuates receptor-mediated signaling leading to NFκB activation. However, no reports exist that have examined antiinflammatory activities of GTE on TNFR1 and TLR4 pro-inflammatory pathways during NASH. We therefore hypothesized that dietary GTE treatment would reduce TLR4 and TNFR1-mediated activation of NFκB that otherwise contributes to liver injury during NASH. To test this, we fed mice an LF or HF diet for 12 wk to induce NASH. Mice then continued on these same diets that were either devoid of, or supplemented with, GTE at a physiologically relevant dietary dose for an additional 8 wk. We then evaluated therapeutic antiinflammatory activities of GTE by assessing histological and biochemical parameters of NASH, hepatic NFκB inflammatory responses, and mRNA expression of TNFR1 and TLR4 receptor complexes along with concentrations of ligands for these receptors.

4.3. Materials and methods

4.3.1. Materials

All solvents and chemicals were HPLC-grade or higher and were purchased from Thermo Scientific. Powdered GTE was kindly provided by Unilever BestFoods. Its composition was verified by HPLC-UV to contain 30% total catechins (w/w) with the following proportions of specific catechins: 48% EGCG, 31% EGC, 13% ECG, 8% EC [220].

4.3.2. Mouse study

The protocol for this study was approved by the Institutional Animal Care and Use Committee at The Ohio State University. Male C57BL6/J mice (5 wk old; n = 46) were purchased from The Jackson Laboratory. Following 1 wk acclimation to the
environmentally-controlled facility, equal numbers of mice were housed individually and randomized to receive an LF or HF diet for 12 wk to induce NASH as we described [16, 25], which was verified histologically and biochemically from a subset of mice in each group (n = 3/treatment). Then, the remaining mice (n = 10 mice/treatment) were randomized to continue on the respective LF or HF diets that were devoid of, or supplemented with, GTE for an additional 8 wk to assess the antiinflammatory therapeutic activity of GTE on NFκB activation. The LF (#D12450J; Research Diets) and HF (#D12492) diets contained 10% and 60% of energy from fat, respectively, and were formulated to contain powdered GTE at 0% or 2% (w/w) as we detailed previously [16]. GTE at 2% closely approximates intakes from epidemiological studies suggesting that green tea (≥10 servings/d) lowers the risk of liver injury and inflammation [139]. This dietary level was also chosen based on our evidence that it lowers NFκB-mediated inflammation during HF-induced NASH [16, 25].

Mice had free access to food and water throughout the entire 20 wk study. Body mass was measured weekly and food intake daily. After the 8 wk GTE treatment period, mice were sacrificed in the fasted state (10 h) under isoflurane anesthesia. Blood was collected from the retroorbital sinus and centrifuged to obtain serum. Liver and adipose tissue (epididymal and retroperitoneal depots; the sum of these masses is defined as total adipose tissue) were excised, washed with phosphate buffered saline, blotted, snap-frozen in liquid nitrogen, and stored at -80°C. The small intestine was also excised, dissected into thirds to represent the duodenum (upper third), jejunum (middle third), and ileum (lower third), and then washed, snap-frozen, and stored at -80°C. Separate portions of the central hepatic lobe from each mouse were also collected into
formalin and into RNAlater® (Sigma-Aldrich) to preserve samples for the assessment of histological parameters of NASH and gene expression, respectively.

4.3.3. Histological Assessment of NASH

Formalin-fixed, paraffin-embedded liver sections (4-5 μm) were stained with hematoxylin and eosin as we described [25] to assess NASH using an established histological scoring criteria [199]. In brief, steatosis was scored as grade 0 for fatty infiltrates occupying <5% hepatic parenchyma, grade 1 for 5%-33%, grade 2 for 33-66%, or grade 3 for >66%. Hepatocyte ballooning was scored as grade 0 (none), grade 1 (few ballooned cells), or grade 2 (predominant ballooning). Hepatic inflammation was scored as grade 0 (none), grade 1 (1 foci/field), grade 2 (2-4 foci/field), or grade 3 (>4 foci/field).

4.3.4. Metabolic Markers

To corroborate histologic findings of NASH, hepatic total lipid was extracted, determined gravimetrically, and solubilized as we described [14] to measure triglyceride, cholesterol (Pointe Scientific) and FFA (Wako Diagnostics) using spectrophotometric kits. Serum lipids were measured directly with these kits. Serum ALT activity and glucose were also measured using clinical assays (Pointe Scientific), and insulin by ELISA (Crystal Chem). Insulin resistance was calculated from fasting concentrations of glucose and insulin using the homeostasis model assessment of insulin resistance (HOMA-IR) as described [221]. The lipid peroxidation biomarker MDA was measured from liver samples by HPLC-FL as we described [25]. MDA was normalized to hepatic protein that was determined using Pierce BCA Protein Assay Kit (Thermo Scientific). Serum TNFα was measured by ELISA (BD Biosciences) following manufacturer’s instructions.
4.3.5. Serum Endotoxin

Serum endotoxin was measured using a commercially available fluorometric assay (PyroGene rFC; Lonza) according to the manufacturer’s instructions. In brief, serum was diluted 1:100 in endotoxin-free water. Following incubation (70°C, 10 min), samples were mixed with the provided working reagent containing recombinant Factor C (rFC). Sample fluorescence was then measured at 380\text{nm}/420\text{nm} (excitation/emission) prior to and after an incubation period (37°C, 1 h) that allows for endotoxin-mediated activation of rFC and subsequent cleavage of a fluorogenic substrate. Serum endotoxin concentration is then determined from the change in sample fluorescence using an endotoxin standard curve that was prepared in parallel.

4.3.6. RT-PCR

Gene expression studies were performed in liver and small intestines as we described [25]. In brief, total RNA was extracted using Trizol (Invitrogen). cDNA was synthesized using a reverse transcription kit (Bio-Rad). RT-PCR analysis was performed using a SYBR Green PCR kit on a Bio-Rad CFX384 instrument (Bio-Rad). All primers were purchased from Sigma-Aldrich (Table 3). Hepatic expression of genes involved in NFκB-mediated inflammation, and signaling through the TLR4 and the TNFR1 receptor complexes, were quantified relative to HPRT using the \(2^{-\Delta\Delta CT}\) method [200]. Expression of genes encoding TJPs were also assessed from the upper, middle, and lower segments of the small intestines to consider site-specific changes in small intestinal permeability consistent with evidence from NAFLD patients that small intestinal bacteria overgrowth contributes to endotoxemia [222].
4.3.7. Western Blotting

Livers were homogenized in iced-cold Pierce T-PER buffer containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) to obtain total cellular proteins [25], which were denatured under reducing conditions. Protein expression studies were performed using an automated Western blotting system according to the manufacturer’s instructions (Protein Simple Wes). Following capillary-based electrophoresis, primary antibodies against phosphorylated-p65 (phospho-p65; 1:10; #3033), p65 (1:50; #8242), β-actin (1:50; #4967; Cell Signaling Technology), and myeloid differentiation 88 (MyD88; 1:200, #ab2064; Abcam) were infused into capillaries to probe target proteins prior to protein visualization using a horseradish peroxidase secondary antibody (ProteinSimple; #PS-MK14). Protein expression was quantified using Compass Software (ProteinSimple) and normalized to that of β-actin (loading control).

4.3.8. Statistical Analysis

Data (means ± SEM) were analyzed using GraphPad Prism (version 6). A Student’s t-test was used to evaluate differences in biomarkers between LF and HF groups following the initial 12 wk feeding period. Two-way ANOVA was used to evaluate main and interactive effects of dietary fat and GTE. Newman-Keuls post-test was used to assess group mean differences following statistically significant main or interactive effects. Bartlett’s test was used to assess equal variances for each study variable. Those not meeting assumptions for equality were log-transformed to achieve equal variances prior to performing analysis by 2-way ANOVA. Pearson correlation ($r_p$) analysis was used to evaluate associations between continuous variables whereas
Spearman correlation ($r_s$) analysis was used to define pair-wise relations between discrete variables. All analyses were considered statistically significant at $P \leq 0.05$.

4.4. Results

4.4.1. HF-feeding induces NASH

Following 12 wk feeding with the LF or HF diets, a subset of mice ($n = 3$/group) were sacrificed to confirm the presence of NASH prior to initiating GTE treatment. Consistent with our prior studies [25], the HF group compared with the LF controls had greater ($P < 0.05$) body mass (45.9 ± 0.7 vs. 24.7 ± 1.3 g), total adipose mass (3.91 ± 0.38 vs. 0.67 ± 0.10 g), and liver mass (1.60 ± 0.18 vs. 0.89 ± 0.34 g). The HF group also had increased ($P < 0.05$) serum ALT activity (53.6 ± 17.3 vs. 7.1 ± 0.3 U/L) and hepatic triglyceride (123.1 ± 14.3 vs. 54.8 ± 13.8 µmol/g liver) compared with LF controls. The latter was corroborated by histological evidence showing increased steatosis and hepatocyte ballooning (Figure 1). Thus, HF feeding induced biochemical and histological evidence of NASH in association with an obese phenotype, which permitted testing of the hypothesis that GTE functions to serve as a therapeutic antiinflammatory strategy for NASH.

4.4.2. GTE treatment ameliorates obesity, liver lipid accumulation, and insulin resistance without affecting energy intake.

At termination of the 20 wk study, data in Table 4 show that mice fed the HF diet compared with those fed the LF diet had greater ($P < 0.05$) body, adipose and liver masses, and concentrations of hepatic triglyceride, cholesterol, and serum FFA. Serum glucose and insulin were also higher in the HF group, and corresponded with increased HOMA-IR. GTE in mice fed an LF diet did not affect any of these parameters. However, GTE treatment in mice fed an HF diet lowered body mass as well as liver and adipose
masses compared with HF controls, which occurred without any GTE-mediated alterations in energy intake ($P>0.05$). GTE treatment in mice fed the HF diet also decreased hepatic concentrations of triglyceride and cholesterol, which were reduced to concentrations not significantly different from LF controls ($P>0.05$). Serum insulin and HOMA-IR were also reduced by GTE in HF-fed mice compared with HF controls, which occurred without affecting serum glucose concentrations.

4.4.3. GTE treatment decreases histological and biochemical evidence of liver injury

Livers from all mice showed little to no visual evidence of inflammatory infiltrates, nor were any between-treatment effects detected ($P>0.05$; Figure 12). However, HF-feeding markedly increased visual evidence of liver steatosis and hepatocyte ballooning compared with LF controls. These parameters were unaffected by GTE in mice fed the LF diet ($P>0.05$). In contrast, GTE treatment in mice fed the HF diet reduced liver steatosis to the extent observed in LF controls whereas hepatocyte ballooning was reduced compared with HF-fed mice ($P<0.05$), and remained higher than that observed from LF mice.

Serum ALT activities and hepatic MDA concentrations were higher in HF-fed mice compared with LF-fed controls (Table 4). Serum ALT and hepatic MDA were both unaffected by GTE in mice fed the LF diet ($P>0.05$). However, GTE treatment in mice fed the HF diet compared with HF controls reduced serum ALT activity to levels not significantly different from LF mice. Hepatic MDA was also reduced by GTE in HF-fed mice, but their concentrations remained higher than those in LF-fed mice. Nonetheless, hepatic MDA concentrations were highly correlated with serum ALT activity ($r_p = 0.69$; $P<0.0001$). Corroborating these findings were observations that liver steatosis and
hepatocyte ballooning scores were each correlated ($P<0.05$) with hepatic MDA ($r_s = 0.46$ and 0.75, respectively) and serum ALT ($r_s = 0.56$ and 0.51, respectively).

### 4.4.4. GTE ameliorates NFκB-mediated inflammation associated with NASH

Consistent with NFκB activation contributing to liver injury during NASH [16], mice fed the HF diet had increased ($P<0.05$) hepatic protein levels of phospho-p65 compared with those of LF mice, which occurred without affecting total p65 expression (Figure 13A). Phospho-p65 and total p65 were unaffected by GTE in LF-fed mice ($P>0.05$). In contrast, GTE treatment in mice fed the HF diet decreased ($P<0.05$) phospho-p65 protein to levels not significantly different from those observed in LF-fed mice ($P>0.05$), which also occurred without any GTE-mediated changes in total p65 protein expression ($P>0.05$).

Hepatic antiinflammatory activities mediated by GTE treatment were corroborated by examining the expression of NFκB-dependent target genes (Figure 13B). Consistent with increased phospho-p65 protein levels, mice fed the HF diet had increased mRNA expression of TNFα and iNOS compared with LF controls. These pro-inflammatory responses were unaffected by GTE treatment in LF-fed mice. In HF-fed mice, however, GTE treatment lowered ($P<0.05$) mRNA expression of both TNFα and iNOS, which were reduced to levels not different from those in LF-fed mice ($P>0.05$).

To support the concept that targeting inflammation is important for treating NASH, correlation analysis was performed to define associations between NFκB-mediated inflammatory responses and biomarkers of hepatic injury. We observed that hepatic phospho-p65 protein levels were correlated ($P<0.05$) with serum ALT ($r_p = 0.46$) and hepatic MDA ($r_p = 0.57$). Similarly, hepatic TNFα and iNOS mRNA levels were also correlated with serum ALT ($r_p = 0.50$ and 0.50, respectively; $P<0.005$) and hepatic MDA.
Collectively, the observed antiinflammatory activities of GTE on NFκB activation and its downstream targets are consistent with our prior findings showing that prophylactic administration of GTE protects against NASH by limiting NFκB-mediated inflammation [16]. However, the specific upstream target(s) by which GTE treatment functions to inhibit NFκB activation during NASH remains unknown, and is the focus of the studies presented below.

4.4.5. GTE lowers ligand availability and expression of TNFR1

Consistent with our hypothesis that GTE decreases NFκB activation during NASH by attenuating receptor-mediated signaling, we assessed hepatic mRNA expression of TNFR1, and its adaptor proteins (RIP1 and TRADD), and serum protein levels of its ligand TNFα. Mice fed the HF diet had greater mRNA expression of hepatic TNFR1 and RIP1 compared with LF controls (Figure 14A). In mice fed the LF diet, GTE had no effect on mRNA expression of TNFR1 and RIP1. However, GTE treatment in mice fed the HF diet reduced TNFR1 and RIP1 expression to levels not different from those of LF controls. In contrast, mRNA levels of hepatic TRADD was unaffected by both HF feeding and GTE (P>0.05).

TNFα is expressed downstream of NFκB activation, but it also functions as a ligand to activate TNFR1/NFκB signaling [223]. Serum concentrations of TNFα were increased in response to the HF diet (Figure 14B). Although GTE had no effect on serum TNFα levels in mice fed the LF diet (P>0.05), it significantly lowered serum TNFα in HF-fed mice compared with those of HF controls (P<0.05). Thus, GTE lowers both the availability of ligand for receptor binding and expression of TNFR1 itself. However,
whether these responses occur upstream or downstream of NFκB activation cannot be determined in the present study. To consider this line of inquiry, correlation analysis was performed to define the relation between NFκB activation and TNFR1 signaling. Contrary to our hypothesis, hepatic phospho-p65 protein levels were not correlated \( (P>0.05) \) with hepatic TNFR1 mRNA levels or serum concentrations of TNFα.

4.4.6. GTE lowers TLR4 ligand availability and receptor complex expression

Our studies also considered the role of GTE functioning to limit TLR4-mediated activation of NFκB (Figure 15). HF-feeding increased \( (P<0.05) \) mRNA expression of hepatic TLR4, its co-receptors (MD2 and CD14), and its adaptor protein MyD88 (Figure 15A). In mice fed the LF diet, GTE had no effect on the expression of these genes. In contrast, GTE treatment in mice receiving the HF diet resulted in lower mRNA expression of TLR4, MD2, and CD14. TLR4 can activate NFκB through MyD88-dependent and -independent pathways [99]. Our findings indicate that GTE lowered MyD88 mRNA and protein (Figure 15A-B) that were otherwise increased in mice fed the HF diet. Moreover, GTE in HF mice lowered MyD88 mRNA and protein expression to levels not significantly different from LF controls.

We also considered that GTE may attenuate TLR4/NFκB-mediated inflammation by limiting ligand availability for TLR4. Consistent with others [224], HF-feeding increased serum concentrations of endotoxin by 3-times compared with LF controls (Figure 15C). GTE in mice fed the HF diet significantly reduced serum endotoxin, but these concentrations remained higher than those observed in LF controls. GTE treatment had no effect on serum endotoxin in mice fed the LF diet. In support of GTE in regulating TLR4/NFκB-mediated inflammation, phospho-p65 protein levels were correlated \( (P<0.05) \) with serum endotoxin concentrations \( (r_p = 0.48) \), hepatic TLR4
mRNA ($r_p = 0.54$), and MyD88 mRNA ($r_p = 0.61$). Serum endotoxin and hepatic mRNA levels of TLR4 and MyD88 were also correlated with serum ALT ($r_p = 0.64-0.76$; $P<0.0001$). Collectively, these observations suggest that GTE may limit inflammation and liver injury during NASH by lowering TLR4 complex expression and availability of endotoxin that are otherwise expected to promote hepatic TLR4-mediated activation of NFkB.

4.4.7. GTE increases intestinal TJP expression otherwise decreased by HF feeding

That GTE improved endotoxemia directed our attention to assess mRNA expression of TJPs in the duodenal, jejunal, and ileal segments of the small intestine to define potential site-specific changes in gut permeability, consistent with small intestinal bacteria overgrowth contributing to endotoxemia in NAFLD patients [222]. In the duodenum, mRNA expression of occludin (OCC) and zonula occluden-1 (ZO-1) was lowered ($P<0.05$) in mice fed the HF diet compared with LF controls whereas claudin-1 (CLDN-1) mRNA was unaffected (Figure 16A). GTE in HF-fed mice restored mRNA expression of both OCC and ZO-1 to levels not significantly different from LF mice. In the jejunum, neither the HF diet nor GTE affected mRNA expression levels of these TJPs (Figure 16B). In contrast, mRNA expression of OCC and CLDN-1 in the ileum were lowered in response to the HF diet, and restored by GTE to levels not different from LF mice (Figure 16C). mRNA expression of ZO-1 in the ileum was unaffected by the HF diet or GTE treatment. Correlation analysis indicated an inverse correlation between serum endotoxin and OCC mRNA in both the duodenum ($r = -0.47$; $P<0.05$) and ileum ($r = -0.51$; $P<0.01$).
4.5. Discussion

This study demonstrates that dietary GTE is an effective treatment for HF-induced NASH consistent with a mechanism of decreasing TNFR1- and TLR4-mediated NFκB activation that otherwise contributes to liver injury. GTE lowered hepatic protein levels of phospho-p65, the transcriptionally active form of NFκB, without affecting total p65 expression. This was accompanied by GTE-mediated decreases in hepatic TNFR1, RIP1, and TNFα mRNA expression and serum TNFα protein concentrations. GTE also lowered serum endotoxin and decreased the expression of hepatic TLR4 complex genes (TLR4-MD2-CD14-MyD88) and MyD88 protein levels. In addition, GTE restored mRNA expression of small intestinal TJs, specifically duodenal OCC and ZO-1 and ileal OCC and CLDN-1, which were otherwise decreased by HF feeding. Greater OCC expression was also correlated with lower serum endotoxin concentrations. Collectively, these findings support a GTE-mediated antiinflammatory mechanism along the gut-liver axis that limits the translocation of intestine-derived endotoxin and decreases hepatic TLR4 and TNFR1 signaling otherwise contributing to NFκB activation and liver injury in NASH.

Effective lifestyle approaches are needed to manage NASH because there are no US Food and Drug Administration-approved treatments for this disorder. Epidemiological evidence suggests that dietary green tea (≥10 servings/d) lowers the risk of hyperlipidemia, liver injury and inflammation [139]. This is supported by studies in NASH patients showing that GTE improves hepatic steatosis and liver function tests [10, 11]. Evidence from genetic- and diet-induced rodent models of NASH also show that green tea and its catechins exert hypolipidemic and antiinflammatory activities when administered in a prophylactic manner [9]. In contrast, the present study, which
was conducted in a therapeutic model of NASH, provides novel evidence that GTE is an effective treatment for HF-induced NASH in mice. Prior studies in the obese (ob/ob) mouse model of NASH show that GTE at 0.5% had limited benefit in protecting against NFκB-dependent pro-inflammatory responses during NASH [15]. In contrast, GTE at 1% attenuates NFκB-mediated inflammation during NASH in both ob/ob mice [13, 15] and in rodents fed an HF diet [16]. Despite these beneficial effects, GTE at 1% does not routinely normalize NFκB-mediated inflammatory responses to levels observed in lean controls [15] whereas the benefits of GTE at 2% were more pronounced [16]. Indeed, findings from the present study showed that GTE at 2% ameliorates histological evidence of NASH and decreases NFκB-mediated inflammatory responses in association with decreases in markers of hepatic injury and lipid peroxidation.

NFκB centrally regulates inflammation and is activated in livers in both patients [75] and rodents with NASH [76, 218, 225]. The importance of decreasing NFκB activation is emphasized by our observations that serum ALT and hepatic MDA are positively correlated (P<0.05) with TNFα and iNOS expression. This is consistent with our prior studies in HF-induced NASH models showing that GTE protects against hepatic NFκB activation [16, 25] and the expression of its downstream pro-inflammatory proteins, by decreasing IκBα phosphorylation [16]. A clear commonality between these studies is that anti-inflammatory activities of GTE, both prophylactically and therapeutically, regulate NFκB activation and related oxidative stress responses. Although considerable study, including our own [16], has focused on defining GTE on pro-inflammatory responses downstream of NFκB activation, the present study aimed to define the upstream mechanism by which GTE attenuates NFκB activation during NASH, which has yet to be reported.
Intracellular ROS, and extracellular signaling from TNFR1 and TLR4, are implicated in inducing NFκB activation in NASH [219]. Our early evidence [15] suggested that GTE lowered ROS to limit NFκB activation by upregulating Nrf2-dependent antioxidant defenses. However, as we showed in Nrf2-KO mice fed an HF diet, prophylactic administration of GTE prevented NFκB activation independent of Nrf2 status [25]. Further study in this model provided support for the current investigation by demonstrating that GTE decreases hepatic TNFR1 and TLR4 mRNA expression regardless of Nrf2 status [25]. The present study extends these observations by showing that GTE lowers hepatic NFκB activation in association with decreasing mRNA levels of TNFR1 and its adaptor protein RIP1 along with circulating TNFα, a ligand for TNFR1. We also showed that GTE lowered the expression of genes comprising the TLR4 complex (TLR4-MD2-CD14-MyD88) along with MyD88 protein levels and circulating levels of endotoxin. Phospho-p65 levels were also decreased by GTE, but without any GTE or HF effects on total p65 expression. This supports an antiinflammatory activity of GTE on IKK-mediated NFκB activation that limits signaling through TNFR1 and TLR4 rather than GTE downregulating NFκB p65 expression or upregulating its degradation.

NFκB is known to transcriptionally regulate TNFR1 expression [226]. Thus, our findings suggest that GTE lowers TNFR1 expression by limiting the transcriptional activities of NFκB. In contrast, TLR4 functions as a pathogen recognition receptor for bacterial constituents [227]. Specifically, TLR4 signaling is activated following its ligation by LPS, a gut-derived endotoxin [228], which triggers NFκB-mediated inflammation. PU.1 and specificity protein 1 (Sp1) transcriptionally upregulate the expression of TLR4 and CD14, respectively [229, 230]. PU.1 deficient mice have lower
hepatic TLR4 expression and are resistant to LPS-induced NFκB activation [231], but whether GTE downregulates TLR4 in a PU.1-dependent manner requires study. Similarly, EGCG inhibits Sp1 activity in vitro [232, 233], but the extent to which this occurs in NASH has not been reported. However, TLR4 signaling increases NFκB activation in both MyD88-dependent and -independent pathways [99]. Our data show that GTE lowers MyD88 protein levels consistent with a lowering of MyD88 mRNA expression. Although this supports GTE in limiting TLR4/MyD88-dependent NFκB activation, it does not preclude the possibility that GTE decreases TLR4-mediated NFκB activation through the MyD88-independent pathway. Determining the mechanism by which GTE lowers MyD88 expression was not examined in the present study. However, signal transducer and activator of transcription (STAT)-1 and -3 bind to the MyD88 promoter region [234]. In vitro, EGCG inhibits IFNγ-induced phosphorylation of STAT-1 [185] and IFNγ upregulates MD2 expression [235]. EGCG also prevents hepatic STAT-3 phosphorylation in obese mice administered a hepatotoxicant [236]. Clearly, the complex interplay of transcriptional events on TLR4 signaling that are potentially regulated by GTE during NASH warrant further study.

Both loss-of-function TLR4 mutant mice and TNFR1-KO mice are protected from diet-induced NAFLD [96, 167]. Our data support GTE in limiting TNFR1- and TLR4-mediated NFκB activation. However, the present study cannot discriminate between independent or additive antiinflammatory effects of GTE on lowering NFκB activation through these pathways. Interestingly, we observed that phospho-p65 protein levels were correlated (P<0.05) with TLR4 mRNA and endotoxin concentrations, but not with serum TNFα or TNFR1 mRNA levels (P>0.05). Because TNFα [237] and TNFR1 [226] are regulated by NFκB, we speculate that that GTE initially suppresses TLR4-mediated
activation of NFκB. In turn, this would be expected to attenuate TNFα expression that is required to induce TNFR1 signaling. In support, GTE in HF-fed mice decreased circulating endotoxin and increased the expression of TJPs, specifically duodenal OCC and ZO-1 and ileal OCC and CLDN-1. This suggests a hepatic antiinflammatory mechanism mediated along the gut-liver axis by which GTE normalizes hepatic NFκB activation by limiting the availability of a gut-derived ligand for TLR4 and pro-inflammatory signaling through this receptor.

Increased endotoxin is implicated in hepatic NFκB activation in NASH patients [92]. Although EGCG mitigates IFNγ-induced increases in epithelial permeability in vitro [185], no reports exist examining catechins on gut barrier function in rodent models (regardless of NASH) despite precedent for other flavonoids (e.g. genistein, quercetin) to improve intestinal barrier function [238]. Our study supports GTE in lowering endotoxemia by restoring intestinal TJP expression. This is of potential therapeutic importance consistent with NASH patients having small intestinal bacteria overgrowth [115]. However, the stability of catechins decreases in a pH-dependent manner such that ~20% of catechins are recovered following simulated digestion [239]. This suggests that in the lower pH region of the small intestine (i.e. duodenum), GTE catechins would be available to directly regulate intestinal barrier function, as suggested in vitro [185]. In contrast, the higher pH of the ileum would be expected to favor catechin metabolite generation. The role of catechin metabolites on TJP expression has not been studied. However, catechin metabolites (e.g. dihydroxyphenyl-γ-valerolactone, phenylpropionic acid) are known to exert a prebiotic effect [240]. This is consistent with catechin metabolite-rich fermented GTE improving NASH symptoms while restoring the composition of gut microbiota that was otherwise
disrupted in mice fed an HF diet [136]. Alternatively, and perhaps complementary, catechins may exert antimicrobial activity on Gram-negative bacteria [241], in which LPS comprises 75% of the outer membrane [94]. Thus, it is plausible that GTE, either directly through its catechins or indirectly through endogenously generated catechin metabolites, lowers endotoxemia during NASH by enhancing TJP expression and/or attenuating microbial dysbiosis to improve gut barrier function.

Although our findings suggest that GTE limits NFκB activation by attenuating the expression of TLR4 and its ligand, we acknowledge that other pathways could be involved. For example, studies in cultured macrophages show that epigallocatechin gallate (EGCG) upregulates Toll-interacting protein (Tollip), an inhibitor of TLR4 signaling, by interacting with the 67kDa laminin receptor (67LR) [177]. SFA, especially palmitate, are also known to activate TLR4 signaling [179]. Although the present study showed no GTE-mediated effects on serum FFA, prior studies demonstrate that GTE lowers hepatic SFA in an HF-model of NASH [144]. GTE was also shown to lower NADPH oxidase activity and peroxynitrite-mediated damage in obese mice with NASH [13]. This supports the possibility that GTE inhibits TLR4 trafficking and assembly, which requires peroxynitrite that is generated downstream of NADPH oxidase activation [178].

In conclusion, our findings support an antiinflammatory activity of GTE on NFκB activation that is mediated along the gut-liver axis to limit hepatic TNFR1 and TLR4 expression while also lowering the systemic availability of TNFα and gut-derived endotoxin. Future studies are needed to define the independent, tissue-specific effects of GTE in decreasing NFκB inflammatory responses that contribute to liver injury in NASH. This is expected to be of public health importance consistent with the growing
recognition that the gut-liver axis is a therapeutic target in NASH [242]. Thus, translational studies examining GTE in NASH patients should be considered, consistent with green tea being considered safe when ingested as recommended [243] and its potential role to effectively mitigate NASH in association with obesity.
Figure 11. Histological evaluation of hematoxylin and eosin-stained liver sections from mice fed an LF or HF diet for 12 wk.

A) Representative liver sections (original magnification 200x) in mice fed an LF or HF diet. B) Histological score of liver steatosis, hepatocyte ballooning, and inflammatory infiltrates. Data (means ± SEM, n = 3 mice per group) were analyzed using an unpaired Student’s t-test to evaluate group differences (P<0.05).
Figure 12. Histologic evaluation of livers from mice fed an LF or HF diet containing GTE at 0 or 2% for 8 wk.

A) Liver sections were stained with hematoxylin and eosin, and representative images were captured at 200x. B) Liver steatosis, hepatocyte ballooning, and inflammatory infiltrates were scored from representative images. Data (means ± SEM, n = 10 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different (P<0.05). NS, no statistically significant effects due to HF or GTE or their interaction were detected (P>0.05). Abbreviations: GTE, green tea extract; HF, high-fat; LF, low-fat.

84
Figure 13. Protein expression of hepatic NFκB p65 and mRNA expression of hepatic pro-inflammatory genes in mice fed an LF or HF diet containing GTE at 0 or 2% for 8 wk.

A) Representative Western blot of phospho-p65, total p65, and the loading control β-actin that were measured from liver homogenates using a capillary-based automated Western blotting system. B) Hepatic mRNA expression of NFκB-dependent target pro-inflammatory genes. RNA was isolated using Trizol and reverse transcribed for RT-PCR analysis for TNFα and iNOS using the primers described in Table 3. Data (means ± SEM, n = 10 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups not sharing a common letter are significantly different (P<0.05). NS, no statistically significant effects due to HF or GTE or their interaction were detected (P>0.05). Abbreviations: GTE, green tea extract; HF, high-fat; iNOS, inducible nitric oxidase synthase; LF, low-fat; phospho-p65, phosphorylated p65 subunit of NFκB; TNFα, tumor necrosis factor-α.
Figure 14. mRNA expression of TNFR1 complex genes and circulating concentrations of TNFα in mice fed an LF or HF diet containing GTE at 0 or 2% for 8 wk.

A) Hepatic mRNA expression of TNFR1, and its adaptor proteins RIP1 and TRADD. RNA was isolated using Trizol and reverse transcribed for RT-PCR analysis using the primers described in Table 3. B) Serum TNFα was measured by ELISA. Data (means ± SEM, n = 10 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups not sharing a common letter are significantly different (P < 0.05). NS, no statistically significant effects due to HF or GTE or their interaction were detected (P > 0.05). Abbreviations: GTE, green tea extract; HF, high-fat; LF, low-fat; RIP1, receptor interacting protein 1; TNFR1, tumor necrosis factor receptor-1; TRADD, TNFR-associated death domain.
Figure 15. Hepatic expression of TLR4 complex genes and circulating concentrations of endotoxin in mice fed an LF or HF diet containing GTE at 0 or 2% for 8 wk.

A) Hepatic mRNA expression of genes comprising the TLR4 receptor complex. RNA was isolated using Trizol and reverse transcribed for RT-PCR analysis for TLR4, its co-receptors MD2, CD14, and its adaptor protein MyD88 using the primers described in Table 3. B) Representative Western blot of hepatic MyD88 and the loading control β-actin that were measured from liver homogenates using a capillary-based automated Western blotting system. C) Serum endotoxin was measured using a fluorometric assay. Data (means ± SEM, n = 10 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups not sharing a common letter are significantly different (P<0.05). Abbreviations: GTE, green tea extract; TLR4, Toll-like receptor 4; MD2, myeloid differentiation 2; CD14, cluster of differentiation 14; MyD88, myeloid differentiation 88; LF, low-fat; HF, high-fat.
Figure 16. mRNA expression of small intestinal TJs in mice fed an LF or HF diet containing GTE at 0 or 2% for 8 wk.

The small intestine was dissected into thirds to represent the duodenum (upper third), jejunum (middle third), and ileum (lower third). mRNA expression of TJs in the: A) duodenum, B) jejunum, and C) ileum. RNA was isolated using Trizol and reverse transcribed for RT-PCR analysis for occludin, ZO-1, and claudin-1 using the primers described in Table 3. Data (means ± SEM, n = 10 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Continued
Figure 16 continued
Groups not sharing a common letter are significantly different ($P<0.05$). NS, no statistically significant effects due to HF or GTE or their interaction were detected ($P>0.05$). Abbreviations: CLDN-1, claudin-1; GTE, green tea extract; HF, high-fat; LF, low-fat; OCC, occludin; TJPs, tight junction proteins; ZO-1, zona occluden-1.
Table 3. Primers used for RT-PCR gene expression studies in Chapter 4.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>GGAAGCCAGAGAACACCCTGC</td>
<td>CCAGAAGCAACAGCAACAG</td>
</tr>
<tr>
<td>CLDN-1</td>
<td>GTTTCAGAGACCCCCTCAGC</td>
<td>AGAAGCCAGGATGAAACCCA</td>
</tr>
<tr>
<td>HPRT</td>
<td>GCTGGTGAAAGGACCTCTCT</td>
<td>CACAGGACTGAACACACTG</td>
</tr>
<tr>
<td>iNOS</td>
<td>TTCTGTGGGACTTGTCCCTGTAG</td>
<td>TGAAGAAAAACCCCTTTGTGCT</td>
</tr>
<tr>
<td>MD2</td>
<td>GACGCTGCTTTCTCCTGCTA</td>
<td>CATTGGTTCCCCTCAGTTT</td>
</tr>
<tr>
<td>MyD88</td>
<td>CTGGGTGAAGAAGCTACTCCAT</td>
<td>TAGAAACTTGGGAGGTGTG</td>
</tr>
<tr>
<td>OCC</td>
<td>CCCATCTGACTATGTGGAAGA</td>
<td>AAAACCGCTTGTCACTTTT</td>
</tr>
<tr>
<td>RIP1</td>
<td>TGTCATCTAGCGGAGGTTG</td>
<td>TCACCACGTGCTGCTCAG</td>
</tr>
<tr>
<td>TLR4</td>
<td>CCTCTGCTTCTCAGAGACTTT</td>
<td>TGTGGAAGCCTTCTGGATG</td>
</tr>
<tr>
<td>TNFα</td>
<td>CTCCAGCGCGGTGCCTATG</td>
<td>GGGCCATAGAAGCTGAGG</td>
</tr>
<tr>
<td>TNFR1</td>
<td>GGGCAGCTTTACCGCTTCC</td>
<td>GTTTGCCTTACGCGCACACA</td>
</tr>
<tr>
<td>TRADD</td>
<td>CTGCGGTGAGCACAGGTGATCC</td>
<td>CAGTACTAGACTTACGCCAGGC</td>
</tr>
</tbody>
</table>

All primer sequences are for mice. Abbreviations: CD14, cluster of differentiation 14; CLDN-1, claudin-1; HPRT, hypoxanthine-guanine phosphoribosyl transferase; iNOS, inducible nitric oxide synthase; MD2, myeloid differentiation protein 2; MyD88, myeloid differentiation protein 88; OCC, occluden; RIP1, receptor interacting protein 1; TLR4, Toll-like receptor-4; TNFα, tumor necrosis factor-α; TNFR1, tumor necrosis factor receptor-1; TRADD, TNFR-associated death domain; ZO-1, zonula occluden-1.
Table 4. Body composition, food intake, and serum and liver metabolic parameters in mice fed an LF or HF diet containing GTE at 0% or 2% for 8 wk.

<table>
<thead>
<tr>
<th></th>
<th>LF</th>
<th>LF + GTE</th>
<th>HF</th>
<th>HF + GTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Body Mass (g)</td>
<td>29.0 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.0 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.4 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.3 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Final Body Mass (g)</td>
<td>29.4 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>27.0 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>47.8 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.5 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Energy Intake (kcal/d)</td>
<td>9.8 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.0 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.0 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.7 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver Mass (g)</td>
<td>1.01 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.90 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.99 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.16 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Adipose Mass (g)</td>
<td>1.20 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.75 ± 0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.29 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.74 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hepatic Total Lipid (mg/g liver)</td>
<td>118.6 ± 9.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>104.3 ± 7.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>284.2 ± 16.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>118.6 ± 9.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hepatic Triglyceride (μmol/g liver)</td>
<td>56.3 ± 2.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43.0 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>159.6 ± 17.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.2 ± 7.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hepatic Cholesterol (μmol/g liver)</td>
<td>8.6 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.4 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.1 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.1 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum FFA (mEq/L)</td>
<td>0.89 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.88 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.09 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.01 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum Glucose (mmol/L)</td>
<td>13.4 ± 0.4&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>12.6 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.3 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.6 ± 0.5&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum Insulin (pmol/L)</td>
<td>68.9 ± 13.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>69.8 ± 14.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>307.7 ± 42.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>176.8 ± 31.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>5.7 ± 1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.1 ± 1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>41.4 ± 9.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.4 ± 3.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum ALT (U/L)</td>
<td>17.1 ± 2.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.0 ± 2.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>112.9 ± 8.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.7 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hepatic MDA (pmol/mg protein)</td>
<td>521.8 ± 46.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>563.7 ± 48.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1240.0 ± 87.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>835.4 ± 49.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Initial body mass represents the body mass of mice after the initial 12 wk dietary treatment period where mice received either an LF or HF diet that was devoid of GTE. Total adipose mass represents the sum of epididymal and retroperitoneal fat pads. Data are means ± SEM, n = 10 in each group. Data were analyzed by 2-way ANOVA with Newman-Keuls post-test to assess group mean differences. Means in a row not sharing a common letter are significantly different, P≤0.05. Abbreviations: ALT, alanine aminotransferase; FFA, free fatty acids; GTE, green tea extract; HF, high-fat; HOMA-IR, homeostasis model assessment-insulin resistance; LF, low-fat; MDA, malondialdehyde.
CHAPTER 5
GREEN TEA EXTRACT PROTECTS AGAINST HEPATIC NFκB ACTIVATION ALONG THE GUT-LIVER AXIS IN DIET-INDUCED OBESE MICE WITH NONALCOHOLIC STEATOHEPATITIS BY REDUCING ENDOTOXIN AND TLR4/MYD88 SIGNALING

5.1. Abstract

GTE reduces NFκB-mediated inflammation during NASH. We hypothesized that its antiinflammatory activities would be mediated in a TLR4-dependent manner. WT and loss-of-function TLR4-mutant (TLR4m) mice were fed an HF diet supplemented with powdered GTE at 0 or 2% for 8 wk before assessing NASH, NFκB-mediated inflammation, TLR4 and its adaptor proteins MyD88 and TRIF, circulating endotoxin, and intestinal TJP mRNA expression. TLR4m mice had lower (P<0.05) body mass compared with WT mice, but similar adiposity, whereas body mass and adiposity were lowered by GTE regardless of genotype. Liver steatosis, serum ALT, and hepatic lipid peroxidation were also lowered by GTE in WT, and were similarly lowered in TLR4m mice regardless of GTE. Phosphorylation of the NFκB p65 subunit and pro-inflammatory genes (MCP-1, iNOS, MPO) were lowered by GTE in WT mice, and did not differ from the lowered levels in TLR4m mice regardless of GTE. TLR4m mice had lower TLR4 mRNA, which was also lowered by GTE in both genotypes. TRIF expression was unaffected by genotype and GTE, whereas MyD88 was lower in mice fed with GTE regardless of genotype. Serum endotoxin was similarly lowered by GTE
regardless of genotype. Duodenal and jejunal CLDN-1 mRNA expression was unaffected in TLR4m mice, and GTE increased CLDN-1 expression in both genotypes; claudin-1 at both sites was inversely correlated with endotoxin (r = -0.58 to -0.43; \( P<0.05 \)). Thus, GTE protects against inflammation during NASH, likely by limiting gut-derived endotoxin translocation and TLR4/MyD88/NFκB activation.

5.2. Introduction

NAFLD is the most common liver disease in the United States where it afflicts 80-100 million individuals [27]. Liver steatosis is the most prevalent form of NAFLD, but can progress in severity to NASH [199], fibrosis, cirrhosis, and potentially hepatocellular carcinoma or liver failure [1]. Tragically, the prevalence of NAFLD is expected to increase due to the high rate of obesity and the lack of validated therapies beyond weight management [217], which has a poor long-term success rate [8].

The pathogenesis of NASH can be described by the “multiple-hit” mechanism [50] in which numerous events including insulin resistance, gut dysbiosis, and hepatic inflammation act in a concerted manner to induce NASH. NFκB-mediated inflammation is a significant contributor to NASH by provoking insulin resistance, mitochondrial dysfunction, and oxidative stress [139]. That NFκB is implicated in “multiple-hits” suggests that limiting its activation would be an effective strategy to ameliorate NASH. Indeed, a pharmacological NFκB inhibitor mitigated liver steatosis, injury, inflammation, and fibrosis in an obese mouse model of NASH [244], but the utility of this therapeutic approach is limited by its adverse effects [245]. This supports a need for safe dietary approaches that limit NFκB to prevent NASH development.

NFκB is activated following IκB phosphorylation, which results in the translocation of NFκB to the nucleus where it upregulates pro-inflammatory gene expression (e.g.
Although NFκB activation is mediated through several mechanisms [219], extracellular signaling through Toll-like receptor-4 (TLR4) is implicated in NAFLD [95]. Ligands for TLR4 include gut-derived endotoxin (e.g. LPS) and SFA [95]; both of which are increased in rodent models of NASH [247]. Thus, strategies that limit ligand availability and/or inhibit hepatic TLR4 signaling would be expected to prevent NFκB-mediated inflammation in NASH.

High dietary intakes of green tea lower the risk of hyperlipidemia, inflammation, and liver injury [139]. Controlled trials in NASH patients show that GTE improves liver function tests and hepatic steatosis [10, 11]. A mechanistic basis for the health benefits of GTE or its polyphenolic catechins has been provided by studies in rodent models of NASH [9]. Indeed, we showed that GTE mitigates liver injury by decreasing DNA binding activity of the NFκB p65 subunit, likely by preventing IκBα phosphorylation [16]. GTE also decreased hepatic phosphorylated-p65 (phospho-p65) without affecting total p65 expression and downregulated NFκB-dependent pro-inflammatory gene expression [25].

The upstream mechanism by which GTE limits NFκB activation during NASH remains unclear. Oxidative stress contributes to NFκB activation. However, GTE decreased hepatic oxidative stress and NFκB activation in a manner that is independent of Nrf2 [25], a cytoprotective transcription factor that upregulates endogenous antioxidant defenses. Further studies in this model [26] and separate rodent studies [25] supported that GTE lowers NFκB activation by limiting pro-inflammatory signaling mediated by tumor necrosis receptor-1 (TNFR1) and Toll-like receptor-4 (TLR4). The expression of TNFR1 and its ligand TNFα are upregulated by NFκB [40], whereas the promoter of TLR4 lacks an NFκB binding site. Thus, GTE likely
decreases TLR4 signaling initially to protect against NFκB-dependent inflammation during NASH. This is also consistent with GTE decreasing circulating endotoxin in association with improving the expression of intestinal TJPs [25]. Thus, we hypothesized that GTE would protect against hepatic NFκB activation in a TLR4-dependent manner to limit hepatic injury during NASH. To test this, loss-of-function TLR4-mutant (TLR4m) mice and wild-type (WT) controls were fed an HF [248] diet devoid of, or supplemented with, GTE for 8 wk. We then assessed NASH, hepatic NFκB inflammation, circulating endotoxin, and expression of intestinal TJPs, and hepatic TLR4 and its adaptor proteins.

5.3. Materials and methods

5.3.1. Materials

All solvents were HPLC-grade and purchased from Thermo Scientific. Powdered GTE was provided by Unilever BestFoods. Its composition was verified by HPLC-UV [220] to contain 30% total catechins (w/w) with the following proportions of specific catechins: 48% EGCG, 31% EGC, 13% ECG, 8% EC.

5.3.2. Study design

This study was approved by the Institutional Animal Care and Use Committee at The Ohio State University. Loss-of-function TLR4m mice (C3H/HeJ; n = 20) and C3H/HeOuJ WT mice (n = 20) at 4 wk of age were purchased from The Jackson Laboratory. TLR4m mice were selected for these studies because they are unresponsive to endotoxin due to a mutation in the Toll/interleukin-1 receptor domain of TLR4 [249] that prevents TLR4/MyD88-dependent signaling [250]. Following 1 wk acclimation to the temperature-, light-, and humidity-controlled facility, TLR4m and WT mice were randomized to receive an HF diet (#D12492; Research Diets) for 8 wk that
contained 60% of energy from fat and formulated with 0 or 2% GTE (w/w) as we detailed [15]. We showed that GTE at 2% exhibits antiinflammatory activities on NFκB activation in obese models of NASH [16, 25, 144, 251]. GTE at 2% is also consistent with epidemiological studies suggesting that green tea (10 servings/d) protects against liver injury and inflammation [139].

Mice had free access to food and water throughout the 8 wk intervention. Body mass was measured weekly and food intake daily. At study termination, mice were euthanized in the fasted state (10 h) under isoflurane anesthesia. Blood was collected from the retro-orbital sinus and centrifuged to obtain serum. Liver and adipose tissue (subcutaneous, epididymal, and retroperitoneal; the sum of these masses is reported as total adipose mass) were excised, washed with phosphate buffered saline, blotted, snap-frozen in liquid nitrogen, and stored at -80°C. The small intestine was excised, dissected into thirds to represent the duodenum (upper third), jejunum (middle third), and ileum (lower third), and then washed, snap-frozen, and stored at -80°C. Separate portions of liver from the central hepatic lobe were collected into formalin and RNAlater® (Sigma-Aldrich) to perform histologic assessment and qPCR studies, respectively, as described below.

5.3.3. Histological analysis of NASH

Paraffin-embedded liver sections (4-5 μm) were stained with hematoxylin and eosin to assess NASH (liver steatosis, hepatocyte ballooning, inflammatory infiltrates) as we described [26] using established criteria [199]. In brief, steatosis was scored based on the percentage of hepatic parenchyma occupied by fatty infiltrates (grade 0 for <5%, 1 for 5%-33%, 2 for 33-66%, or 3 for >66%). Ballooning was scored as: grade 0 for none, 1 for few ballooned cells, or 2 for predominant ballooning. Inflammation was
scored based on the number of inflammatory infiltrate foci: grade 0, grade 1 (1 foci/field), grade 2 (2-4 foci/field), or grade 3 (>4 foci/field).

5.3.4. Biochemical assessment of NASH, oxidative Stress, and endotoxemia

Hepatic total lipid was extracted and solubilized as we described [14] to assess triglyceride, cholesterol (Pointe Scientific), and FFA (Wako Diagnostics) using spectrophotometric kits in accordance with the manufacturer’s instructions. Serum lipids were determined directly with these kits. Serum ALT activity and glucose were measured using clinical assays (Pointe Scientific), and insulin by ELISA (Crystal Chem). Insulin resistance was then calculated from fasting glucose and insulin concentrations using the homeostasis model assessment of insulin resistance (HOMA-IR) [221]. Hepatic MDA, a biomarker of lipid peroxidation, was determined by HPLC-FL as we described [15]. Reduced (GSH) and oxidized (GSSG) glutathione were measured to calculate hepatic glutathione redox status (GSH/GSSG) and total glutathione [tGSH; defined as GSH + 2(GSSG)] by HPLC-ECD as we described [252]. MDA and glutathione were normalized to hepatic protein, which was determined using a Pierce BCA Protein Assay kit (ThermoFisher Scientific). Serum endotoxin was measured using a fluorometric kit (PyroGene rFC; Lonza) as we detailed [25].

5.3.5. qPCR

The expression of genes involved in hepatic inflammation and gut integrity were measured by qPCR as we described [26]. In brief, total RNA was extracted with Trizol (Invitrogen), cDNA was synthesized using an iScript kit (Bio-Rad) and qPCR was performed using a CFX384 instrument (Bio-Rad) with a SYBR Green PCR kit (Bio-Rad). Primers were purchased from Sigma-Aldrich (Table 5). Target gene expression
was normalized to hypoxanthine-guanine phosphoribosyl transferase using the $2^{-\Delta\Delta CT}$ method [253].

5.3.6. Western Blotting

Hepatic proteins were extracted using Pierce T-PER buffer containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Following protein denaturation under reducing conditions [26], phospho-p65 and total p65 expression were measured using a Protein Simple WES automated Western blotting system as we described [25]. In brief, following capillary-based electrophoresis, primary antibodies against phospho-p65 (1:10; #3033), p65 (1:50; #8242), and β-actin (1:50; #4967; Cell Signaling Technology) were infused to probe target proteins prior to protein visualization using a horseradish peroxidase secondary antibody (ProteinSimple; #PS-MK14) and quantified with Compass Software (ProteinSimple). Conventional Western blotting was performed to determine the expression of MyD88 (1:250, #ab2064; Abcam) and β-actin (1:1000) using primary antibodies diluted in 5% Tris-buffered saline (TBS)-milk and the expression of TRIF (1:1000, #ab13810, Abcam) using primary antibody diluted in TBS. After washing in TBS, membranes were incubated with IRDye® 800CW secondary antibody (1:10000; P/N925-32211; Li-Cor) with quantification accomplished using a Li-Cor Fc imaging station. Each target protein was normalized to that of β-actin (loading control).

5.3.7. Statistical analysis

Data (means ± SEM) were analyzed using GraphPad Prism (version 6.0). Two-way ANOVA was used to evaluate main and interactive effects of GTE and genotype. Newman-Keuls post-test was used to assess group mean differences following statistically significant main or interactive effects. Prior to 2-way ANOVA, variances
were assessed for equality using Bartlett’s test and those not meeting assumptions for equal variance were log-transformed. Pearson correlation (r) analysis was used to define pair-wise relationships between study variables. All analyses were considered statistically significant at $P\leq0.05$.

5.4. Results

5.4.1. GTE protects against obesity in TLR4m mice without affecting insulin resistance.

At study termination, TLR4m mice were shown to have gained less body mass than WT controls ($P<0.05$; Table 6). Dietary GTE supplementation, regardless of genotype, limited body weight gain to a similar extent. Total adipose mass was not different ($P>0.05$) between TLR4m and WT mice, but adiposity was lowered to a similar extent by GTE regardless of genotype (Table 6). Although serum FFA was unaffected by genotype, it was increased in WT and TLR4m mice that received GTE (Table 6). A genotype x GTE interaction ($P<0.05$) indicated that HOMA-IR was lower in TLR4m mice regardless of GTE, whereas it was lowered to a greater extent by GTE in WT mice compared with TLR4m mice (Table 6). Lower HOMA-IR in TLR4m mice was attributed to lower serum glucose and insulin concentrations compared with WT controls. However, the more substantially lowered HOMA-IR by GTE in WT mice occurred due to a more pronounced attenuation in glucose, but not insulin, compared with TLR4m mice (Table 6). Thus, these data suggest that GTE protects against obesity independent of intact TLR4 signaling, whereas maximal GTE-mediated improvements in insulin resistance may require functional TLR4, consistent with evidence that TLR4 signaling suppresses insulin sensitivity [254].
5.4.2. GTE and loss-of-TLR4 signaling similarly attenuate NFκB activation during NASH.

WT mice provided GTE had lower protein levels of hepatic phospho-p65 compared with WT controls (Figure 17A). Phospho-p65 levels were also lower in TLR4m mice compared with WT mice, which were not significantly different from those in WT mice fed GTE. GTE in TLR4m mice had no additional effect on hepatic phospho-p65. Neither GTE nor TLR4m mice had any effect on total p65 expression (Figure 17A).

The antiinflammatory activities of GTE and loss-of-TLR4 signaling were corroborated by examining NFκB-dependent pro-inflammatory gene expression (Figure 17B). Consistent with lower NFκB phosphorylation, WT mice fed GTE had decreased mRNA expression of hepatic iNOS, MPO, and MCP-1 compared with those of WT controls. Similarly, expression of each pro-inflammatory gene was lower in TLR4m mice regardless of GTE, and did not differ from those of WT mice fed GTE. These data suggest that GTE lowers NFκB-mediated inflammation in WT mice to the extent attributed to the loss of TLR4 signaling in TLR4m mice.

5.4.3. GTE inhibits MyD88-dependent TLR4 signaling

We measured hepatic TLR4 expression to examine the extent to which GTE prevents NFκB activation by limiting TLR4 signaling (Figure 18). Compared to WT controls, GTE in WT mice resulted in significantly lower hepatic TLR4 mRNA expression. Hepatic TLR4 mRNA in TLR4m mice was also lower than that of WT controls, but not different from WT mice provided GTE. GTE in TLR4m mice further decreased TLR4 mRNA expression relative to TLR4m mice.

Based on these findings, we examined whether GTE attenuates TLR4/NFκB signaling in an MyD88-dependent manner and/or through the MyD88-independent
pathway that requires TRIF-mediated signaling [255]. Protein expression of hepatic MyD88 was unaffected by genotype (Figure 18). However, MyD88 expression was lowered to a similar extent by GTE in both WT and TLR4m mice compared with their respective controls. In contrast, TRIF expression was unaffected by GTE and genotype (Figure 18). Thus, GTE attenuates hepatic NFκB activation consistent with a mechanism of limiting TLR4/MyD88-dependent signaling.

5.4.4. GTE lowers TLR4 ligand availability regardless of genotype

Our data suggest that GTE attenuates NFκB activation by limiting the expression of TLR4. However, it may also limit hepatic TLR4 signaling by decreasing the availability of gut-derived endotoxin, which is the major component of the outer membrane of Gram-negative bacteria [94]. Serum endotoxin was unaffected by genotype (Figure 19A). However, endotoxin concentrations were similarly decreased by GTE in both WT and TLR4m mice. We considered that GTE protected against endotoxemia by enhancing gut barrier function (Figure 19B). mRNA levels of the intestinal TJP claudin-1 were unaffected by GTE and genotype in the ileum. However, claudin-1 expression in the duodenum and jejunum was similarly higher in response to GTE in both WT and TLR4m mice. We also observed that serum endotoxin was inversely correlated with duodenal and jejunal claudin-1 expression ($r_p = -0.58$ to $-0.43$, $P<0.05$), and positively correlated with expression levels of TLR4 and phosphorylated-p65 ($r_p = 0.46-0.57$; $P<0.05$). These data suggest that GTE protects against endotoxemia by limiting gut permeability, and that these intestinal-level benefits are mediated independent of intact TLR4 signaling.
5.4.5. GTE ameliorates oxidative stress and liver injury during NASH.

Based on GTE lowering NFκB activation to the extent observed in TLR4m mice (Figure 17), we hypothesized that its antiinflammatory benefits would also limit liver injury and oxidative stress (Figure 20). Compared with WT controls, serum ALT was similarly lower in WT mice fed GTE and in TLR4m mice fed no GTE. GTE had no additional benefit on serum ALT in TLR4m mice (Figure 20A). Data also show that hepatic MDA was lower in WT mice fed GTE. MDA was also lower in TLR4m mice, but without any additive effect of GTE (Figure 20B). Similarly, hepatic glutathione redox status (i.e. GSH/GSSG; Figure 20C) was increased by GTE in WT mice and in TLR4m mice fed no GTE; GTE had no additive benefit in TLR4m mice. GTE-mediated improvements in GSH/GSSG in WT mice were explained by increased hepatic GSH without affecting GSSG. In contrast, TLR4m mice had lower GSH (Figure 20D), but a more substantial lowering of GSSG concentrations (Figure 20E) that resulted in an increased GSH/GSSG ratio (Figure 20C). Thus, tGSH was lower in TLR4m mice compared to WT controls (Figure 20F), and GTE increased tGSH regardless of genotype. We also observed that hepatic GSH/GSSG was inversely correlated with hepatic TLR4 mRNA, and protein expression levels of MyD88 and phospho-p65 ($r_p$ = -0.44 to -0.60; $P<0.01$ for each), suggesting that decreases in hepatic inflammation function to preserve glutathione redox status.

We also examined the relation between serum ALT and hepatic oxidative stress, consistent with evidence that liver injury in NASH is mediated by oxidative stress [16, 26]. In agreement, serum ALT was correlated with hepatic MDA ($r_p$ = 0.48, $P<0.01$) and inversely related with hepatic GSH/GSSG ($r_p$ = -0.37, $P<0.05$). Thus, antiinflammatory
activities of GTE may protect against oxidative stress-mediated liver injury during NASH.

5.4.6. GTE ameliorates liver steatosis and injury during NASH.

NFκB-dependent inflammation is implicated in liver steatosis [256]. Thus, we examined the interactive effects of GTE and TLR4 signaling on histological parameters of NASH (Figure 21). Regardless of genotype or GTE treatment, livers from all mice showed little to no visible evidence of inflammatory infiltrates. In contrast, WT mice fed no GTE had marked evidence of macrovesicular steatosis and hepatocellular ballooning. GTE in WT mice protected against steatosis and hepatocellular ballooning to levels no different from TLR4m mice; GTE had no effect in TLR4m mice. These findings were corroborated by directly assessing hepatic lipids (Table 6). In agreement, compared with WT controls, hepatic total lipid and triglyceride concentrations were similarly lower in WT mice fed GTE and TLR4m mice, but unaffected by GTE in TLR4m mice.

5.5. Discussion

This study demonstrates that GTE protects against HF-induced NASH consistent with a mechanism involving the gut-liver axis that limits metabolic endotoxemia and hepatic TLR4/MyD88-mediated NFκB activation. Consistent with our hypothesis, GTE attenuated hepatic NFκB activation, oxidative stress, and NASH to the extent attributed to the loss of intact TLR4 signaling in TLR4m mice. GTE also decreased the expression of MyD88, but not TRIF, suggesting that it prevented TLR4/MyD88-mediated NFκB activation. Contrary to our hypothesis, GTE attenuated endotoxemia by increasing TJP expression independent of intact TLR4 signaling. Thus, GTE likely limits NFκB
activation in a TLR4-dependent manner at the liver, but intestinal-level benefits that limit endotoxin translocation are mediated independent of intact TLR4 signaling.

No pharmacological treatments exist for NASH [257], thereby emphasizing lifestyle approaches to decrease the risk of liver-related morbidity and mortality. Although weight loss could be effective, high rates of weight regain [8] indicate a need for alternative dietary strategies. Indeed, high intakes of GTE are associated with lower risks of inflammation and liver injury [139]. GTE in NASH patients also improves liver function tests and ultrasound evidence of liver steatosis [10, 11], although the latter requires histologic confirmation. The therapeutic efficacy of GTE in NASH is supported by preclinical studies showing that GTE ameliorates NASH in a prophylactic [9] and therapeutic [25] manner by attenuating NFκB activation. Although the present study examined GTE during NASH induction, the findings are also likely to have therapeutic application.

Our strategy to target NFκB activation during NASH is consistent with its involvement in insulin resistance [258] and oxidative stress [139]. GTE limits NFκB inflammation in preclinical models of NASH [25, 251] by decreasing IκBα phosphorylation [16]. However, IκBα phosphorylation is provoked by intracellular ROS and/or extracellular signaling from TNFR1 and TLR4 [219]. Although GTE lowered inflammation in association with upregulating endogenous antioxidant defenses [15], studies in Nrf2-knockout mice showed that GTE protects against NFκB activation in an Nrf2-independent manner [26]. This essentially rules out ROS-lowering activities of GTE to decrease NFκB inflammation. In contrast, GTE limited NFκB activation in association with lower TNFR1 and TLR4 mRNA expression and availability of their respective ligands [25, 251]. However, TLR4 and its ligand are regulated independent
of NFκB [259] whereas TNFR1 and TNFα are NFκB-dependent [226, 237]. Thus, GTE likely attenuates TNFR1 signaling secondary to decreased TLR4/NFκB activation (Figure 22), which provides rationale in the present study to examine TLR4-dependent antiinflammatory activities of GTE.

We used TLR4m mice lacking intact TLR4/MyD88-dependent signaling [250] to test whether GTE limits NFκB activation in a TLR4-dependent manner. This approach was utilized because mice overexpressing TLR4 have heightened sensitivity to endotoxin-induced sepsis [260]. Data show that GTE in WT mice prevented NFκB-dependent inflammation to the extent observed in TLR4m mice. These antiinflammatory activities occurred in association with GTE lowering TLR4 expression. TLR4m mice also had lower TLR4 mRNA levels that were additionally lowered by GTE, but without any further decrease in NFκB activation. This supports that TLR4 expression is regulated, at least in part, independent of NFκB activation. Indeed, NFκB induces activator protein-1 [259] to upregulate TLR4 [261]. The transcription factor PU.1 also upregulates TLR4 [261], but whether GTE attenuates PU.1 during NASH has not been reported. Thus, studies examining GTE on PU.1-mediated TLR4/NFκB inflammation are warranted.

Hepatic TLR4 signaling is mediated through the adaptor proteins MyD88 and TRIF, which regulate “early” and “late” NFκB activation, respectively (Figure 24) [99]. MyD88-dependent signal transduction occurs immediately following cell surface binding of LPS [262]. In contrast, TRIF-dependent signaling first requires endocytosis of LPS [262], which facilitates endotoxin clearance [101]. Neither GTE nor genotype affected TRIF expression, whereas GTE lowered MyD88 and circulating endotoxin regardless of genotype. This suggests that GTE attenuates “early” NFκB activation in a
TLR4/MyD88-dependent manner while lowering endotoxemia independent of promoting hepatic LPS clearance. MyD88 expression is regulated by signal transducer and activator of transcription 1 (STAT1) [234]. Thus, GTE potentially attenuates TLR4/NFκB inflammation by downregulating STAT1, consistent with EGCG preventing IFNγ-mediated phosphorylation of STAT1 in vitro [263], but this requires investigation during NASH.

SFA and endotoxin both function as the ligands for TLR4 [95]. Although not tested directly, we show that serum FFA was increased by GTE regardless of genotype. Others have shown that SFA in the hepatic FFA fraction are unaffected by GTE [144]. However, we show that GTE lowered circulating endotoxin regardless of genotype. This occurred without any apparent involvement of TRIF-mediated LPS clearance. We therefore examined intestinal TJP expression consistent with gut barrier dysfunction contributing to endotoxemia during NASH [264]. GTE increased duodenal and jejunal claudin-1 expression regardless of genotype. This is consistent with GTE increasing small intestinal TJP expression and EGCG preventing IFNγ-induced epithelial permeability in vitro [185]. Circulating endotoxin was also inversely correlated with claudin-1 expression, and positively correlated with hepatic TLR4 and phospho-p65 expression. Thus, GTE likely functions in a TLR4-independent manner to prevent the translocation of gut-derived endotoxin by limiting intestinal permeability, which would otherwise provoke hepatic TLR4/NFκB signaling.

GTE likely prevents TLR4/NFκB activation by limiting endotoxin availability. Future studies are warranted to examine whether GTE limits endotoxemia by mitigating gut microbial dysbiosis, which occurs in both patients and rodents with NASH [265]. Indeed, GTE catechins or microbial-derived catechin metabolites exert antimicrobial activity on
Gram-negative bacteria found in the gut microbiota [240, 241]. Fermented GTE also ameliorates gut microbial dysbiosis otherwise induced by an HF-diet in association with improving NASH symptoms [136]. Thus, future studies are needed to examine whether GTE-mediated improvements in gut health protect against NASH.

Prior study shows that GTE protects against oxidative stress and NFκB-mediated inflammation during NASH and that the antiinflammatory activities of GTE are not mediated through its Nrf2-dependent antioxidant effects [25]. Since NFκB activation exacerbates oxidative stress [266], we considered that the antiinflammatory activities of GTE would protect against oxidative stress-mediated liver injury. We show that GTE lowered hepatic MDA and serum ALT to the levels observed in TLR4m mice, suggesting that decreases in TLR4/NFκB signaling by GTE likely protect against oxidative stress-mediated liver injury. In addition, GTE and loss-of-TLR4 signaling similarly improve glutathione redox status as evidenced that GTE in WT mice increased hepatic GSH/GSSG to the levels observed in TLR4m mice. This indicates that GTE protects against hepatic oxidative stress likely by limiting TLR4/NFκB activation.

Although GTE and loss-of-TLR4 signaling improve inflammation and hepatic redox status to a similar extent, their respective benefits on glutathione metabolism are apparently mediated through differing mechanisms. Increased GSH/GSSG in TLR4m mice was attributed to lower GSSG concentrations without improving GSH levels, consistent with evidence that TLR4 mice have lower GSH levels than WT mice [267]. In contrast, GTE in WT mice improved GSH/GSSG by increasing GSH without affecting GSSG, consistent with GTE upregulating GSH biosynthesis by increasing mRNA expression of GCLc [15, 16]. However, GTE in TLR4m mice had no additive benefit on GSH/GSSG, despite GTE-mediated increases in tGSH status that were reflected by
increased GSH. This provides evidence that GTE-mediated protection against hepatic oxidative stress is not mediated through its TLR4-dependent antiinflammatory activities. Collectively, GTE exerts both hepatic antioxidant and antiinflammatory activities during NASH. However, these concurrent effects are not mediated through each other.

NFκB activation contributes to NASH by increasing fatty acid uptake and impairing VLDL export. Our study also considered that the antiinflammatory activities of GTE would protect against liver steatosis. Histological evidence clearly showed that liver steatosis in WT mice fed GTE was attenuated to the extent observed in TLR4m mice consistent with similar decreases in NFκB inflammation in these mice. Additional study is warranted to define the lipid-lowering activities of GTE that are mediated in a TLR4-dependent manner, consistent with GTE lowering the expression of genes relating to fatty acid uptake and lipogenesis, and upregulating those involved in β-oxidation [25, 268].

Data also show that HF-induced NFκB activation occurred with little to no evidence of inflammatory infiltrates. This observation is consistent with earlier studies in HF- and genetic-induced obese models of NASH [14-16, 25, 251], and suggests that GTE mitigates hepatic NFκB activation at hepatocytes rather than resident Kupffer cells. Thus, future investigations should consider the cell-specific antiinflammatory activities of GTE. In conclusion, GTE protects against liver injury during NASH by limiting gut-derived endotoxin translocation and hepatic TLR4/MyD88-dependent signaling that induces NFκB inflammation. These findings are consistent with the recognition that the gut-liver axis is a therapeutic target in NASH [264]. Thus, translational studies examining GTE treatment on metabolic endotoxemia and consequent TLR4/NFκB-mediated liver injury should be considered, consistent with green tea being recognized to be safe when used as recommended [243].
Figure 17. Hepatic protein expression of NFκB p65 and mRNA expression of NFκB-dependent pro-inflammatory genes in WT and TLR4m mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

A) Representative blot of phospho-p65, total p65, and the loading control β-actin measured from liver whole cell homogenates using WES capillary-based automated Western blotting system. B) mRNA expression of hepatic NFκB-dependent pro-inflammatory genes. RNA was extracted with Trizol and reverse transcribed for RT-PCR analysis for iNOS, MPO and MCP-1 using the primers described in Table 5. Data (means ± SEM, n = 10 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups not sharing a common letter are significantly different (P≤0.05). NS, no statistically significant main or interactive effects were detected (P>0.05). Abbreviations: GTE, green tea extract; HF, high-fat; iNOS, inducible nitric oxidase synthase; MCP-1, monocyte chemoattractant protein-1; MPO, myeloperoxidase; phospho-p65, phosphorylated p65 subunit of NFκB; TLR4m, Toll-like receptor 4-mutant; WT, wild type.
Figure 18. Hepatic expression of TLR4 and its adaptor proteins MyD88 and TRIF in WT and TLR4m mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

RNA was extracted with Trizol and reverse transcribed for RT-PCR analysis using the primers described in Table 5. Protein expression of MyD88, TRIF, and β-actin (loading control) were measured from liver whole cell homogenates by Western blotting. Data (means ± SEM, n = 10 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups not sharing a common letter are significantly different (P≤0.05). NS, no statistically significant main or interactive effects were detected (P>0.05). Abbreviations: GTE, green tea extract; HF, high-fat; MyD88, myeloid differentiation 88; TRIF, TIR-domain-containing adapter-inducing interferon-β; TLR4, Toll-like receptor 4; TLR4m, TLR4-mutant; WT, wild type.
Figure 19. Serum endotoxin and mRNA expression of small intestinal claudin-1 in WT and TLR4m mice fed an HF diet containing GTE at 0 or 2% for 8 wk

A) Serum endotoxin was measured using a fluorometric assay. B) The small intestine was dissected into thirds to represent the duodenum (upper third), jejunum (middle third), and ileum (lower third). mRNA expression of claudin-1 in the duodenum, jejunum, and ileum. Data (means ± SEM, n = 10 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups not sharing a common letter are significantly different (P≤0.05). NS, no statistically significant main or interactive effects were detected (P>0.05). Abbreviations: GTE, green tea extract; HF, high-fat; TLR4m, Toll-like receptor 4-mutant; WT, wild type.
Figure 20. Serum liver injury, hepatic lipid peroxidation and glutathione redox status in WT and TLR4m mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

A) Serum ALT was measured spectrophotometrically. B) Hepatic MDA was measured by HPLC-FL. C-F) Hepatic GSH and GSSG were measured by HPLC-ECD to determine GSH/GSSG and tGSH. Data (means ± SEM, n = 10 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups not sharing a common letter are significantly different (P≤0.05). Abbreviations: ALT, alanine aminotransferase; GSH, reduced glutathione; GSSG, oxidized glutathione; GTE, green tea extract; HF, high-fat; tGSH, total glutathione; TLR4m, Toll-like receptor 4-mutant; WT, wild type.
Figure 21. Histologic evaluation of livers from WT and TLR4m mice fed an HF diet containing GTE at 0 or 2% for 8 wk.

A) Liver sections were stained with hematoxylin and eosin, and representative images were captured at 200x. B) Liver steatosis, hepatocyte ballooning, and inflammatory infiltrates were scored from representative images. Data (means ± SEM, n = 10 mice per group) were analyzed by 2-way ANOVA with Newman-Keuls post-test to evaluate main and interactive effects. Groups without a common letter are significantly different (P≤0.05). NS, no statistically significant main or interactive effects were detected (P>0.05). Abbreviations: GTE, green tea extract; HF, high-fat; TLR4m, Toll-like receptor 4-mutant; WT, wild type.
Table 5. Primers used for RT-PCR gene expression studies in Chapter 5.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin-1</td>
<td>GTTTGCAGAGACCCCATCAC</td>
<td>AGAAGCCAGGATGAAACCCA</td>
</tr>
<tr>
<td>HPRT</td>
<td>GCTGGTGAAAAGGACCTCT</td>
<td>CACAGGACTGAACACCTGC</td>
</tr>
<tr>
<td>iNOS</td>
<td>TTCTGTGCTGTCACCAGTGG</td>
<td>TGAAGAAACCCCTTGTGCT</td>
</tr>
<tr>
<td>MPO</td>
<td>CCATGGTCCAGATCATCACA</td>
<td>GCCGGTACTGATTGTTCAGG</td>
</tr>
<tr>
<td>MCP-1</td>
<td>TGATCCCAATGAGTGGCTGGAG</td>
<td>ATGTCTGGACCCATTCCTTCTTG</td>
</tr>
<tr>
<td>TLR4</td>
<td>CCTCTGCTCTTCACTACAGAGACTTT</td>
<td>TGTGGAAGCCTTCCTGGATG</td>
</tr>
</tbody>
</table>

All primer sequences are for mice. Abbreviations: HPRT, hypoxanthine-guanine phosphoribosyl transferase; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein-1; MPO, myeloperoxidase; TLR4, Toll-like receptor-4.
Table 6. Body composition, insulin resistance, and liver lipid in WT and TLR4m mice fed a high-fat diet containing GTE at 0% or 2% for 8 wk.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>WT + GTE</th>
<th>TLR4m</th>
<th>TLR4m + GTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Body Weight</td>
<td>19.9 ± 0.4</td>
<td>19.9 ± 0.4</td>
<td>18.2 ± 0.4</td>
<td>18.2 ± 0.6</td>
</tr>
<tr>
<td>(g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Body Weight</td>
<td>37.9 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.1 ± 1.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33.6 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.5 ± 1.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>(g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver Mass (g)</td>
<td>1.62 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.36 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.09 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.19 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Adipose Tissue</td>
<td>3.67 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.44 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.29 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.52 ± 0.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Glucose (mg/dL)</td>
<td>246.6 ± 9.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.6 ± 9.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>108.8 ± 7.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>93.6 ± 4.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum Insulin (ng/mL)</td>
<td>0.95 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.31 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.44 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.38 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>14.0 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.0 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.7 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hepatic Total Lipid</td>
<td>132.3 ± 7.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100.7 ± 4.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>117.9 ± 8.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100.3 ± 3.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(mg/g liver)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatic Triglyceride</td>
<td>107.6 ± 10.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58.1 ± 2.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69.5 ± 5.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.0 ± 4.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(μmol/g liver)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatic Cholesterol</td>
<td>19.3 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.9 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.4 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.1 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(μmol/g liver)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Triglyceride</td>
<td>97.9 ± 8.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91.1 ± 7.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.9 ± 5.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59.8 ± 7.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Cholesterol</td>
<td>227.0 ± 5.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>208.9 ± 8.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>181.5 ± 6.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>213.5 ± 5.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum FFA (mEq/L)</td>
<td>0.78 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.02 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.96 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Initial body weight represents the body weight of mice before 8 wk dietary treatment. Total adipose mass represents the sum of epididymal, retroperitoneal, and subcutaneous fat pads. Data are means ± SEM, n = 10 in each group. Data were analyzed by 2-way ANOVA with Newman-Keuls post-test to assess group mean differences. Means in a row not sharing a common letter are significantly different, P ≤ 0.05. Abbreviations: ATGL, adipose triglyceride lipase; FFA, free fatty acids; GTE, green tea extract; HOMA-IR, homeostasis model assessment: insulin resistance; HSL, hormone sensitive lipase; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein-1; MPO, myeloperoxidase; WT, wild type; TLR4m, Toll-like receptor 4-mutant.
6.1. Overview

There are no well-established treatments for NASH other than weight loss, which has poor long-term success rate [8]. Thus, effective dietary strategies are needed to mitigate NASH. The underlying premise of this dissertation is supported by epidemiological evidence from Japan suggesting that high dietary intakes of green tea (>10 servings/d) lower the risk for liver injury and inflammation [139]. In support, human clinical trials showed that GTE treats liver steatosis and improves liver function tests in NAFLD patients [10, 11]. Studies in rodents showed that GTE protects against NASH and NFκB-mediated inflammation. However, the mechanism by which GTE exerts its antiinflammatory activities during NASH has not been identified. Thus, this dissertation aimed to define the mechanism by which GTE exerts its antiinflammatory activities against NFκB activation during NASH in diet-induced obese mice.

The central hypothesis was that GTE would protect against NFκB-mediated inflammation by improving Nrf2-dependent antioxidant defenses, and/or by lowering TLR4 and TNFR1 signaling. Contrary to this hypothesis, studies in Nrf2-KO mice (Chapter 3) demonstrated that GTE suppresses NFκB activation independent of Nrf2 status [25]. However, GTE was shown to lower NFκB-mediated inflammatory responses by decreasing ligand availability for, and expression of, TLR4 and TNFR1
receptor complexes that are otherwise increased due to HF feeding (Chapter 4) [26].

In addition, data support that GTE suppresses hepatic NFκB-mediated inflammation by restoring intestinal TJP expression to mitigate gut-derived endotoxin levels [26]. Lastly, studies performed in WT and TLR4m mice (Chapter 5) support that GTE exerts its hepatic anti-inflammatory activities against TLR4/MyD88/NFκB in a TLR4-dependent manner at the liver, and in a TLR4-independent manner at the intestine. Thus, GTE likely protects against hepatic inflammation during NASH along the gut-liver axis. In addition, GTE administered with an HF diet (Chapter 3 and 5) prevents the development of NASH, whereas studies in Chapter 4 demonstrated that GTE can also reverse HF-induced NASH in obese rodents. Thus, the work presented in this dissertation indicates that GTE has both preventive and therapeutic effects in mitigating NASH and NFκB-mediated hepatic inflammation and that its protective effects are mediated through a lowering of TLR4 and TNFR1 signaling independent of Nrf2 status.

6.2. Rodent models of NASH

6.2.1. Diet-induced rodent models of NASH

Diets that are methionine and MCD, choline-deficient amino acid defined (CDAA), high-fructose and HF, are commonly used to induce NASH in rodents. A MCD or CDAA diet lacks choline or methionine, the two amino acids required for phosphatidylcholine synthesis and triglyceride secretion from liver [269]. Thus, these diets induce NASH while decreasing total body mass and adipose mass [270], as well as increasing insulin sensitivity [271]. Rodents fed with a CDAA or MCD diet are usually used to study NASH without insulin resistance or obesity. The absence of obesity and insulin resistance in rodents fed CDAA or MCD diet fail to reflect the fact that the majority of NASH patients
also develop obesity and insulin resistance. Thus, CDAA and MCD diets are not good dietary models of NASH to test our hypothesis.

A high-fructose diet mimics the high-fructose corn syrup-induced NAFLD [272-274]. Although a high-fructose diet induces diabetes and liver steatosis in rodents, it has limited potential to induce inflammation during NASH [275]. Some studies reported that a high-fructose diet successfully induces NASH, but the hepatic inflammation occurs in periportal zone [276], instead of in centrilobular zone that occurs in NASH patients [28]. Thus, high-fructose diet-induced obese rodents are not a good model to study GTE-mediated antiinflammatory activities during NASH because it fails to reflect the severity and/or pattern of hepatic inflammation in NASH patients.

An HF diet containing 45% energy from coconut oil or butter fail to develop simple steatosis after 14 wk of feeding [277]. A lard-based HF diet containing 45% energy from fat induce rapid liver lipid accumulation and insulin resistance within days. However, a lard-based 45% HF diet only induces mild steatosis, instead of NASH as induced by MCD diets [278]. In addition, studies with chronic ingestion of a lard-based 45% HF diet reported a nonlinear pattern of liver fat accumulation with a decrease followed by an increase in liver fat [279]. Thus, a lard-based 45% HF diet is likely not a good candidate to investigate the antiinflammatory activities of GTE during NASH. HF diet with 60% energy from lard has been reported to induce NASH, insulin resistance and obesity in rodents after 8 wk of feeding [280]. Thus, considering the quantity and type of dietary fat, the lard-based HF diet containing 60% energy from fat is more effective in inducing NASH compared to HF diet containing 45% energy from coconut oil/ butter, or lard.
Clearly, the lard-based HF diet containing 60% energy from fat fails to reflect the dietary pattern of NASH patients because the quantity of fat used in an HF diet exceeds the amount of fat needed to induce NASH in humans [281]. However, similar to NASH patients, rodents fed a lard-based HF diet containing 60% energy from fat develop liver steatosis, inflammation, obesity and insulin resistance, which cannot be achieved in other dietary models of NAFLD. Thus, the lard-based HF diet containing 60% energy from fat is likely to serve as the best dietary model of NASH in studying the antiinflammatory activities of GTE during NASH.

6.2.2. Genetic rodent models of NASH

Several genetic-induced obese rodent models of NASH are widely used to study NAFLD. Leptin-deficient mice (ob/ob), leptin-resistant mice (db/db) and obese Zucker rats (fa/fa) rapidly develop obesity and liver inflammation and steatosis. Similar to NASH patients, ob/ob mice develop obesity, insulin resistance, liver steatosis and injury. However, ob/ob mice are leptin-deficient, which is different from high leptin levels in NASH patients [282, 283]. db/db mice and fa/fa rats develop leptin resistance due to the mutation in long-term leptin receptors [282, 284]. However, those two models usually develop fibrosis, which limits the research on inflammatory responses during NASH to mirror NASH patients with little to none fibrosis [284, 285]. These two models are usually used to examine fibrosis or severe insulin resistance and are not good models to study the antiinflammatory activities of GTE during NASH. Collectively, 60% HF-induced obese model of NASH is likely to be the best model to study GTE-mediated antiinflammatory activities in NASH to mirror obese patients with NASH in absence of severe fibrosis.
6.3. Green tea catechins, catechin metabolites, and caffeine are likely responsible for GTE-mediated hepatoprotective activities during NASH

Green tea catechins have low bioavailability due to the relatively low intestinal absorption, and the rapid hepatic biotransformation [286, 287]. Despite their low bioavailability, chronic ingestion of green tea or its catechins protects against NASH pathogenesis in genetic- or diet-induced obese rodents [13-16]. The hypolipidemic, antioxidant and antiinflammatory activities of GTE are usually attributed to its catechins. EGCG, the major green tea catechin, inhibits the pathogenesis of fatty liver disease and metabolic syndrome in obese mice [198]. This supports the favorable hepatoprotective effects due to green tea catechins.

However, catechin metabolites derived from green tea catechins also have immunomodulatory effects. In vivo study demonstrated that the major metabolite of EGCG, 5-(3',5'-dihydroxyphenyl)-γ-valerolactone, increased the activity of CD4⁺ T cells and natural killer cells [288]. Microbial-derived catechin metabolites functions as prebiotics to the gut microbiota [240, 241]. In addition, GTE has caffeine too. Dietary intake of caffeine exerts anti-obese activities by increasing adipose lipolysis [289] and increasing thermogenesis [290]. However, tissue adaption is developed to compensate its effects due to chronic ingestion of caffeine [291-293]. Future studies using each specific catechin, catechin metabolite, or caffeine, are warranted to address the question that which component is responsible for the antiinflammatory and hypolipidemic activities of GTE during NASH.
6.4. GTE exerts both preventive and therapeutic effects against NFκB-mediated inflammation during NASH

GTE functions to inhibit NASH and NFκB-mediated inflammation when administered in a prophylactic manner. Studies in rodents using genetic-induced models of NASH show that GTE or catechins exert hypolipidemic and antiinflammatory activities [9]. Evidence from diet-induced obese models of NASH supports that GTE prevents the development of liver steatosis, injury and oxidative stress that is otherwise induced by HF diet [9]. Studies in WT mice fed an HF diet showing that GTE protects against hepatic NFκB activation [16, 25] and the expression of its downstream proinflammatory proteins by decreasing the phosphorylation of IκBα, the inhibitory protein of NFκB [16]. Consistent with studies showing that GTE functions as a preventive strategy for NASH, work presented in Chapter 3 demonstrated that GTE, when provided with HF diet, inhibits the development of NASH, insulin resistance and NFκB-dependent inflammatory responses. This supports that GTE exerts hypolipidemic, anti-diabetic and antiinflammatory activities when provided in a prophylactic manner.

Studies in Chapter 4 were conducted in a therapeutic model of NASH. This demonstrated that GTE, when provided after the development of NASH that was induced by HF feeding, can reverse NASH and lower NFκB-dependent inflammation. Although other researchers have administered EGCG to mice after 9 wk of HF feeding to test the therapeutic activities of EGCG in NASH and diabetes, EGCG only prevented further increase in blood glucose and fasting insulin due to HF feeding without reversing it [198]. Studies in Chapter 4 compared the NASH parameters before and after 8wk of GTE treatment, and demonstrated that GTE reverses NASH [26]. This provides novel
evidence for the therapeutic effects of GTE during NASH in preclinical studies, and is consistent with human clinical trials showing that green tea and its catechins improve liver function tests in NASH patients [10, 11].

A clear commonality between studies in Chapter 3 and Chapter 4 is that antiinflammatory activities of GTE, both prophylactically and therapeutically, regulate NFκB activation and related oxidative stress responses. This supports that GTE has similar antiinflammatory mechanisms when administered prophylactically or therapeutically. Future studies can focus on prophylactic benefits of NASH to examine the mechanisms of GTE in protecting against obesity-mediated NASH to simply the study design because the effects are generalizable from preventive models to the therapeutic situations. Thus, studies in Chapter 5 were conducted in the simplified “preventive” model to examine the TLR4-dependent antiinflammatory activities of GTE during NASH.

6.5. GTE protects against NFκB activation independent of Nrf2

Consistent with the evidence showing that Nrf2 is a major regulator of liver lipid deposition during NASH [192], Nrf2-KO mice in Chapter 3 showed exacerbated pathology of NASH and liver inflammation compared with WT controls fed an HF diet. This supports that Nrf2 plays an important role in suppressing liver steatosis, oxidative stress and hepatic NFκB activation.

The antiinflammatory activities of GTE in HF-induced obese rats occurred in association with increased concentrations of hepatic glutathione, an antioxidant that is synthesized in an Nrf2-dependent manner [16]. A study in vitro demonstrated that EGCG at physiologically relevant levels upregulates the nuclear accumulation of Nrf2
Further study in Chapter 3 also showed that GTE upregulates the mRNA expression of Nrf2 and its downstream gene Nqo1 in the liver of mice fed an HF diet, supporting that GTE improves the expression and activation of Nrf2 in vivo [25]. However, studies in Chapter 3 demonstrated that GTE limits NFκB activation during NASH in an Nrf2-independent manner [25] (Figure 22), suggesting that the antiinflammatory activities of GTE are unlikely to be attributed to an Nrf2-dependent antioxidant function. In addition, GTE ameliorates obesity, insulin resistance and liver injury regardless of Nrf2 status [25], showing that not only antiinflammatory activities, but anti-obese and anti-diabetic activities of GTE are independent of its Nrf2-mediated antioxidant effects.

Although GTE-mediated protection against NFκB is Nrf2-independent, the antiinflammatory activities of GTE might be mediated through Nrf2-independent antioxidant effects. Antioxidant responses are not solely due to Nrf2-mediated mechanisms. Other antioxidant transcription factors might also upregulate the antioxidant defenses to protect against oxidative stress-induced NFκB activation. For example, both FoxOs and sirtuins play an important role in upregulating antioxidant defenses [294, 295]. FoxO 1/3/4 triple knockout mice have exacerbated liver steatosis, as well as the expression of inflammatory and fibrotic genes in diet-induced NASH [296]. Clinical studies on NASH patients show that sirtuins signaling is lower during NASH [297]. EGCG has been reported to stimulate the phosphorylation of FoxO in vitro [164] and the expression of hepatic FoxO3a and Sirtuin-1 in vivo [298]. Thus, future studies may consider the possibility that the antiinflammatory activities of GTE are mediated
through FoxO-dependent or sirtuins-dependent antioxidant effects using FoxO- or sirtuins-deficient rodents.

Similar to Nrf2, Nrf1 also binds to ARE to regulate lipid metabolism and cellular stress [147]. However, Nrf1 regulates steady-state cellular stress whereas Nrf2 provides secondary defense against unscheduled stress such as HF-induced ROS [147]. Evidence from liver-specific Nrf1-deficient mice support that Nrf1 functions to regulate hepatic lipid metabolism [209]. Nrf1 increases the expression of PPARα coactivator lipin1 [209], and lowers the levels of PPARγ coactivator of PGC-1β [210]. EGCG has been reported to upregulate the mRNA expression of Nrf1 and fatty acid oxidation genes in skeletal muscle [211]. Therefore, GTE might exert its antiinflammatory activities against NFκB activation during NASH by activating Nrf1-dependent cytoprotection. Future studies may also consider investigating the Nrf1-dependent hepatoprotective activities by using hepatic conditional Nrf1 deficient rodents.

6.6. GTE protects against NFκB activation by lowering ligand availability and receptor expression of TLR4 and TNFR1 pathways

Studies in Chapter 3 support that GTE attenuated mRNA expression of TNFR1 and TLR4 in an Nrf2-independent manner [25]. These findings support the possibility that GTE attenuates receptor-mediated signaling leading to NFκB activation. Studies in Chapter 4 provide evidence that GTE lowers the ligand availability and receptor expression of TLR4 and TNFR1 pathway to mitigate NFκB-mediated inflammation during NASH [26, 251]. Studies in Chapter 5 support that GTE exerts its antiinflammatory activities in a TLR4/MyD88-dependent manner.
GTE-mediated inhibition of TLR4 signaling is consistent with the evidence showing that LPS-induced macrophage iNOS activity and expression are inhibited by green tea and its catechins in vitro [21-24]. Green tea consumption also improves human gut health by increasing the proportion of the Bifidobacterium [175], an important probiotic that can improve gut barrier function and reduce intestine LPS [176]. Similarly, GTE-mediated inhibition of TNFR1 signaling is consistent with the evidence showing that GTE decreases hepatic protein expression and adipose mRNA expression of TNFα, the ligand for TNFR1, in ob/ob mice with NASH [15]. EGCG inhibits the formation of TNFR1 receptor complex in mice with TNFα-mediated lung inflammation [171]. A study in vitro also shows that EGCG inhibits TNFR1 protein expression during TNFα-induced inflammation [172]. Thus, GTE and its catechins exert hepatic antiinflammatory activities by inhibiting TLR4 and TNFR1 pathways that otherwise lead to NFκB activation (Figure 22).

6.7. **GTE exerts antiinflammatory activities during NASH along gut-liver axis**

Studies in Chapter 4 showed that GTE lowers hepatic NFκB activation and metabolic endotoxemia by restoring intestinal TJP expression, supporting that the antiinflammatory activities by GTE along gut-live axis. Studies in Chapter 5 demonstrated that GTE exerts its antiinflammatory activities against by lowering TLR4/MyD88-mediated hepatic NFκB activation in a TLR4-dependent manner, and by reducing metabolic endotoxemia and improving gut barrier function in a TLR4-independent manner. This further supports that GTE protects against hepatic inflammation during NASH along the gut-liver axis (Figure 23).
This dissertation only examined the mRNA expression of TJPs without measuring protein expression, assembly and post-translational modification of TJPs. Gut permeability was not directly measured, either. High levels of gut-derived endotoxin might be attributed to two factors, overgrowth of Gram-negative bacteria and impaired gut barrier function. In addition, this dissertation did not examine whether GTE directly improves expression of the intestinal TJPs, or by improving factors that relate to “leaky gut”, such as insulin resistance and gut bacterial dysbiosis. Although studies in Chapter 4 and 5 support the protective effects of GTE along the gut-liver axis, further studies are warranted to examine the gut microbiota and gut barrier function to evaluate the severity of overgrowth, or profile shift of gut microbiota, and gut leakiness due to HF feeding and the protective effects by GTE.

6.8. Limitations and future direction

Although findings in Chapter 3-5 suggest that GTE limits NFκB activation by attenuating the expression of TLR4 and its ligand, I acknowledge that other aspects of TLR4/MyD88 pathway or TLR4-related pathways could be involved. GTE likely inhibits TLR4 signaling via mechanisms other than improving gut integrity and lowering expression of TLR4 receptor complex during NASH. For example, other possibilities should also be considered, such as the TLR4 inhibitory pathway (67LR-Tollip), TLR4 receptor complex assembly, hepatic LPS clearance and TRIF-mediated “late” NFκB activation, SFA-mediated TLR4/NFκB activation, as well as PU.1- and STAT1-mediated regulation of TLR4 and MyD88 expression.
6.8.1. 67LR-Tollip pathway

Studies in cultured macrophages show that EGCG, the major GTE catechin, upregulates Tollip, an inhibitor of TLR4 signaling, by interacting with the 67LR [177]. EGCG failed to exert its antiinflammatory activities against LPS/TLR4-mediated NFκB inflammatory responses in endothelial cells with RNA interference-mediated silencing of 67LR [299] or anti-67LR antibody treatment [177]. In addition, RNA interference-mediated silencing of Tollip also blocked EGCG from exerting its antiinflammatory activities [177]. With the presence of 67LR, EGCG at physiologically relevant levels is able to rapidly upregulate the expression of Tollip [299] to inhibit TLR4 signaling induced by LPS. This supports that GTE or its major catechin EGCG likely protects against TLR4/NFκB-mediated inflammation during NASH by directly binding to 67LR to induce the expression of TLR4 inhibitory protein Tollip and to lower the expression of TLR4.

Tollip is an adaptor protein that directly associates with the cytoplasmic domain of IL-1R, TLR4 and TLR2 and acts as their inhibitory protein [300, 301]. Tollip forms a complex with IL-1 receptor-associated kinase (IRAK) to inhibit the phosphorylation and kinase activity of IRAK. Under the stimulation of IL-1R/TLR, Tollip-IRAK complex is recruited to IL-1R/TLR and the association between Tollip and IL-1R/TLR is mediated through IL-1RAcP, and the co-recruitment of MyD88 to IL-1/TLR triggers the auto-phosphorylation and the activation of IRAK [300]. Tollip is phosphorylated by IRAK when stimulated by LPS [301]. Phosphorylated IRAK dissociates from IL-1R/TLR and phosphorylated Tollip to activate NFκB [300]. Tollip overexpression inhibits IL-1R/TLR-mediated NFκB activation [300]. Although no study has reported the susceptibility of
Tollip-deficient mice to HF-induced live inflammation, Tollip-deficient mice are susceptible to LPS-induced neuroinflammation [302]. This supports that Tollip plays an important role in suppressing LPS-induced TLR4/NFκB inflammation.

EGCG is also able to lower TLR4 expression through 67LR [177]. Tollip contains both ubiquitin-associated domains and endosome-targeting domains, and has been reported to cause the degradation of TGF-β type I receptor via the recruitment of E3 ubiquitin ligases. Thus, EGCG-mediated reduction in TLR4 expression is likely mediated by ubiquitin-degradation of TLR4. Thus, Tollip likely inhibits TLR4 signaling by lowering the activity of IRAK or by increasing ubiquitin-degradation of TLR4. Other than the induction of rapid expression of Tollip, GTE and its catechins might also inhibits the phosphorylation of Tollip and IRAK, as well as increases the recruitment of E3 ubiquitin ligases to induce TLR4 degradation. Thus, future studies are warranted to examine the effects of GTE and its catechins on the expression, cellular trafficking and post-translational modification of 67LR and Tollip in TLR4 inhibitory pathway after LPS stimulation.

6.8.2. TLR4 receptor complex assembly

TLR4 trafficking into lipid rafts in cell membrane requires peroxynitrite and NOX activation [303]. Increased peroxynitrite and NOX activation in rodent models of NASH or NASH patients contributes to TLR4 recruitment and dimerization in the lipid rafts of cell membrane [178]. p47phox deficiency protects mice fed an HF diet from liver steatosis and NFκB-mediated inflammation [178], supporting the TLR4 trafficking into lipid rafts is p47phox-dependent. Scavenging of peroxynitrite also inhibits the progression of NASH in obese rodents [178], supporting that peroxynitrite plays an important role in TLR4
trafficking and dimerization into lipid rafts. Thus, TLR4 receptor complex assembly is NOX-, p47phox-, and peroxynitrite-dependent. GTE was shown to lower NADPH oxidase activity and peroxynitrite-mediated damage in obese mice with NASH [13]. This supports the possibility that GTE and its catechins inhibit TLR4 trafficking to and assembly in cell surface. Energy transfer microscopy imaging and TLR4 receptor receptor-trafficking assays [304] can be conducted to examine whether GTE and its catechins inhibit the TLR4 receptor complex assembly and the trafficking of TLR4 receptor to cell membrane.

6.8.3. Hepatic LPS clearance

Studies in Chapter 4 and 5 showed that GTE lowers TLR4 ligand availability as evidenced by lower serum endotoxin levels. Serum endotoxin levels are increased by endotoxin from the gut into the portal blood, and decreased by the hepatic clearance of endotoxin. Although studies have reported that lung-derived endotoxin can also triggers systemic inflammation, lung-derived endotoxemia usually occurs due to nonprotective ventilator strategies (i.e. high tidal volume and/or low/zero end-expiratory pressure) [305]. Evidence in Chapter 4 and 5 supports that GTE lowered metabolic endotoxemia by restoring intestinal TJP. However, due to the absence of data on endotoxin levels in portal blood, I cannot preclude the possibility that GTE protects against metabolic endotoxemia by accelerating hepatic endotoxin clearance. TRIF-mediated TLR4 signaling requires endotoxin endocytosis (Figure 23).

Data in Chapter 5 showed that the protein expression of TRIF is unaffected by GTE, indicating that GTE failed to affect the endotoxin hepatic clearance. However, whether the expression of TRIF increases with high endotoxin endocytosis is unclear.
In contrast, the recruitment of TRIF to LPS endosome, instead of TRIF expression, might be a better indicator. It is likely that GTE increases the hepatic clearance of endotoxin to increase the recruitment of TRIF to LPS endosome without affecting TRIF expression. However, the recruitment of TRIF to LPS endosome might not be a good indicator for LPS endocytosis, either. If GTE increases the de-phosphorylation and de-acylation of lipid A in LPS to accelerate LPS clearance, the longevity of LPS endosome will be shortened to limit its potential to recruit TRIF.

Indeed, the best way to evaluate the effects of GTE on LPS clearance is to measure the difference of serum endotoxin levels between portal vein and hepatic vein. The effects of GTE on gut endotoxin translocation should be directly evaluated by comparing endotoxin levels in portal blood in mice fed an HF diet with 0% or 2% GTE. The effects of GTE on hepatic LPS clearance can be evaluated by comparing the serum endotoxin levels in portal vein and hepatic vein despite the technical challenge of collecting blood from portal vein and hepatic vein at the same time. Thus, comparing endotoxin levels in portal blood and cardiac blood would be an alternative method to evaluate hepatic LPS clearance.

6.8.4. SFA-mediated TLR4/NFκB activation

SFA, especially palmitate, are also known to activate TLR4 signaling [179]. During obesity-mediated NASH, insulin resistance inhibits triglyceride lipolysis and re-esterification of FFA in adipose tissue to increase FFA flux to the liver. Thus, it is likely that GTE also protects against TLR4/NFκB inflammation by lowering SFA levels. However, studies in Chapter 4 showed no GTE-mediated effects on serum FFA and studies in Chapter 5 showed GTE-mediated increase in circulating FFA consistent with
reduced adipose mass. The major component of adipose-derived FFA is SFA, the ligand for TLR4. Thus, the fact that GTE protects against TLR4/NFκB activation without lowering circulating FFA support that GTE inhibits endotoxin-triggered TLR4/NFκB activation without lowering SFA-triggered TLR4/NFκB inflammation.

Consistent with prior studies demonstrating that GTE lowers hepatic SFA in an HF-model of NASH [144], studies in Chapter 3 and 4 demonstrated that GTE lowers mRNA expression of CD36, the transporter centrally regulating hepatic fatty acid uptake, and genes involved in lipogenesis (SREBP-1c, FAS, SCD1). This indicates that GTE likely lowers the levels of hepatic fatty acids that serve as the ligand for TLR4. The increased FFA due to GTE supplementation as shown in Chapter 5 is likely to be taken by skeletal muscles for fatty acid oxidation in mitochondrial and peroxisome, consistent with the evidence that EGCG improves muscle fatty acid oxidation [306].

6.8.5. GTE might suppress TLR4/MyD88/NFκB signaling by regulating other transcription factors

PU.1 transcriptionally upregulates the expression of TLR4 [229], respectively [229, 230]. PU.1 deficient mice have lower hepatic TLR4 expression and are resistant to LPS-induced NFκB activation [231], but whether GTE downregulates TLR4 in a PU.1-dependent manner requires study. Specificity protein 1 (Sp1) transcriptionally regulates the expression of CD14 [230]. EGCG inhibits Sp1 activity in vitro [232, 233], but the extent to which this occurs in NASH has not been reported. MyD88 expression is centrally regulated by STAT1 [234]. Thus, GTE potentially attenuates TLR4/NFκB inflammation by downregulating STAT1, consistent with EGCG preventing IFNγ-mediated phosphorylation of STAT1 in vitro [263], but this requires investigation during
NASH. Collectively, GTE might protect against TLR4/MyD88-mediated NFκB activation by inhibiting transcription factors (PU.1, Sp1, and STAT1) associated with the expression of TLR4 receptor complex (Figure 24). Future studies can be conducted in knockout models to examine the PU.1-, Sp-1, or STAT1-dependent antiinflammatory activities of GTE.

6.8.6. TLR4-dependent antiinflammatory activities through MyD88 and TRIF by GTE

TLR4m mice lacking intact TLR4/MyD88-dependent signaling [250] were used in Chapter 5 to test whether GTE limits NFκB activation in a TLR4-dependent manner. TLR4m mice are not an ideal model to accurately define the TLR4-dependent hepatoprotective activities against NFκB activation and steatosis by GTE because TLR4m fail to develop NASH despite the intact TLR4/TRIF signaling. However, TLR4m model is effective in defining the TLR4-independent activities by GTE in lowering hepatic TLR4 and MyD88 expression, metabolic endotoxemia, and in improving intestinal TJP expression.

A TLR4 overexpression model is ideal for testing the TLR4-dependence in the antiinflammatory and hypolipidemic activities of GTE. However, mice overexpressing TLR4 have heightened sensitivity to endotoxin-induced sepsis [260] and cannot survive chronic GTE treatment. Thus, in vitro studies with TLR4 overexpression is needed to further test the TLR4-dependent antiinflammatory activities of green tea catechins.

TLR4 mutant mice that have blocked TLR4/MyD88 signaling had little to no evidence of NASH despite the intact TLR4/TRIF signaling. This supports that TLR4/MyD88 signaling plays a pivotal role in NASH pathogenesis. TRIF signaling that
mediated “late” stage of NFκB activation occurs after LPS endocytosis. It is likely that LPS endocytosis requires MyD88-mediated “early” NFκB activation (Figure 23). Thus, the blocked TLR4/MyD88 signaling might also inhibits the TLR4/TRIF signaling and prevents TRIF-mediated TLR4/NFκB activation. In addition, TRIF knockout mice fed a CDAA diet have lower liver steatosis, but exacerbated liver injury, inflammation and fibrosis compared to WT controls. This is consistent with GTE-mediated protection against liver steatosis, but contrary to GTE-mediated antiinflammatory activities. Therefore, inhibition of TRIF does not ameliorates, but exacerbates liver inflammation during NASH. Studies in Chapter 5 supports that GTE protects against NFκB-mediated hepatic inflammation without affecting TRIF.

6.9. Conclusion

GTE, when provided in preventive or therapeutic manner, is able to protect against NFκB-mediated inflammation and NASH. GTE mitigates NFκB-mediated liver injury independent of Nrf2-mediated antioxidant defenses. GTE exerts its antiinflammatory activities against NFκB activation during NASH by lowering ligand availability and receptor expression of TNFR1 and TLR4 pathways. Receptor expression and ligand availability of TNFR1 pathways are transcriptionally regulated by NFκB activation, making TNFR1 pathway a secondary responses to other pathways. Receptor expression and ligand availability of TLR4 pathways are not regulated by NFκB activation and are more correlated with NFκB-mediated inflammatory responses, indicating that TLR4 pathway is likely the initial target of green tea and its catechins.

GTE protects against hepatic NFκB-mediated inflammation along gut-liver axis. GTE mitigates TLR4/NFκB inflammation in a TLR4-dependent manner at the liver, and
improves gut integrity and mitigate metabolic endotoxemia in a TLR4-independent manner at the intestine. TLR4/MyD88 pathway, instead of TLR4/TRIF pathway is the target of GTE to protect against liver inflammation during NASH.

This dissertation provides evidence that GTE functions as a novel dietary preventive and therapeutic strategy for NASH in rodent models. Preclinical studies in this dissertation identify that GTE protects against hepatic NFκB-mediated inflammation along gut-liver axis, establishing the clinical framework in treating NASH patients by targeting TLR4. The outcomes of the studies are of substantial public health significance because they provide basis for translational studies that aim to mitigate NASH. Thus, my dissertation supports future clinical studies of GTE in the management of NASH and obesity. Green tea is considered safe when used as recommended [243]. Although the pharmacological dose and bioactive constituents need further investigation, GTE supplement might be used in combination with or in place of traditional weight management therapy to mitigate NASH.
Figure 22. Green tea extract protects against hepatic NFκB activation during NASH by inhibiting TLR4 and TNFR1 signaling independent of Nrf2-dependent antioxidant defenses.

GTE exerts its hepatic antiinflammatory activities against NFκB activation during NASH independent of its Nrf2-dependent antioxidant activities. GTE treatment inhibits NFκB activation by lowering ligand availability and receptor expression of TLR4 and TNFR1 pathways. Since the expression of TNFR1 and its ligand TNFα is regulated by NFκB whereas the promoter of TLR4 does not have NFκB binding site, GTE-mediated protection against TNFR1 pathway likely occur secondary to GTE-mediated protection against TLR4/NFκB inflammation during NASH.
Figure 23. MyD88- and TRIF-dependent TLR4 signaling that leads to hepatic NFκB activation during NASH.

Following LPS binding to TLR4, MyD88 immediately transduce TLR4 signaling from cell membrane to trigger “early” NFκB activation. LPS-TLR4 undergoes endocytosis and LPS clearance. LPS endosome recruits TRIF, which triggers “late” NFκB activation. GTE exerts its hepatic antiinflammatory activities against NFκB activation during NASH by inhibiting MyD88-dependent TLR4 signaling without affecting TRIF-dependent TLR4 signaling.
Figure 24. GTE/EGCG might suppress TLR4/MyD88/NFκB signaling by regulating other transcription factors.

GTE and its major catechin EGCG protects against hepatic NFκB-mediated inflammation during NASH by lowering gut-derived endotoxin levels and expression of CD14, TLR4 and MyD88. GTE and/or EGCG might lower the expression of CD14, TLR4 and MyD88 by inhibiting Sp1, PU.1 and STAT1 expression and/or transcriptional activities, respectively. GTE protects against hepatic inflammation during NASH along the gut-liver axis.
References


on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. Proc Natl Acad Sci U S A 2008;105:16767-16772.


[153] Choi HK, Pokharel YR, Lim SC, Han HK, Ryu CS, Kim SK, Kwak MK, Kang KW. Inhibition of liver fibrosis by solubilized coenzyme Q10: role of Nrf2 activation in


[159] Li W, Kong AN. Molecular mechanisms of Nrf2-mediated antioxidant response. Mol Carcinog 2009;48:91-104.


161


[224] Pendyala S, Walker JM, Holt PR. A high-fat diet is associated with endotoxemia that originates from the gut. Gastroenterology 2012;142:1100-1101 e1102.


[237] Shakhov AN, Kuprash DV, Azizov MM, Jongeneel CV, Nedospasov SA. Structural analysis of the rabbit TNF locus, containing the genes encoding TNFβ (lymphotoxin) and TNFα (tumor necrosis factor). Gene 1990;95:215-221.


