Nonalcoholic steatohepatitis (NASH) is the most common liver disease in industrialized countries. NASH is a progressive disease that can lead to cirrhosis, cancer, and death, and there are currently no approved therapies. The development of NASH in animal models requires intact TLR9, but how the TLR9 pathway is activated in NASH is not clear. Our objectives in this study were to identify NASH-associated ligands for TLR9, establish the cellular requirement for TLR9, and evaluate the role of obesity-induced changes in TLR9 pathway activation. We demonstrated that plasma from mice and patients with NASH contains high levels of mitochondrial DNA (mtDNA) and intact mitochondria and has the ability to activate TLR9. Most of the plasma mtDNA was contained in microparticles (MPs) of hepatocyte origin, and removal of these MPs from plasma resulted in a substantial decrease in TLR9 activation capacity. In mice, NASH development in response to a high-fat diet required TLR9 on lysozyme-expressing cells, and a clinically applicable TLR9 antagonist blocked the development of NASH when given prophylactically and therapeutically. These data demonstrate that activation of the TLR9 pathway provides a link between the key metabolic and inflammatory phenotypes in NASH.
Hepatocyte mitochondrial DNA drives nonalcoholic steatohepatitis by activation of TLR9

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Introduction

Hepatocyte steatosis and ballooning and inflammation of the liver constitute steatohepatitis, which occurs in response to a diverse range of stressors ranging from excess alcohol to drug-induced liver injury. Obesity-driven metabolic syndrome is the most common reason for steatohepatitis and is termed nonalcoholic steatohepatitis (NASH) (1). Overnutrition and obesity uniformly result in hepatic steatosis, which can progress to steatohepatitis, fibrosis, and cirrhosis. The biological processes promoting progression from steatosis to steatohepatitis are of great interest but are currently poorly understood.

TLR9 is an endosomal pattern recognition receptor (PRR) for which CpG-rich bacterial DNA and mammalian self-DNA are ligands (2). We have identified a requirement for TLR9 in toxic liver inflammation and fibrosis (3, 4), and this has been confirmed for hepatic steatosis and inflammation in the choline-deficient amino acid–defined diet model of NASH (5). Apart from this basic finding of a requirement for TLR9 in the development of NASH, very little is known about the identity of TLR9 ligands and how the TLR9 pathway is activated in NASH. The metabolic changes in NASH suggest that there may be unique interactions with TLR9 biology in NASH, beyond that found for liver injury in general. For example, mitochondrial DNA (mtDNA) has many features that make it a potent ligand for TLR9, and there is an increase in hepatocyte mitochondrial content in NASH (6).

The demonstration of an increase in total liver mtDNA and oxidized DNA (oxDNA) in NASH and the ability of these molecules to activate TLR9 suggest a potential mechanism by which the changes induced by overnutrition result in greater activation of a proinflammatory pathway that leads to a transition from steatosis to steatohepatitis (6, 7).

Results and Discussion

We tested whether hepatocyte nuclear DNA (nDNA) and mtDNA from high-fat diet–induced (HFD-induced) NASH livers are more potent activators of TLR9 than DNA from control livers. Figure 1A shows that mtDNA isolated from hepatocytes of mice fed a HFD for 12 weeks resulted in greater activation of a TLR9 reporter than did mtDNA from mice fed a chow diet (CD). This was confirmed in mouse Kupffer cells (KCs), which demonstrate upregulation of Thfa transcripts in response to hepatocyte mtDNA from HFD-fed mice (Figure 1B).

Hepatocyte mtDNA is increased in NASH and is known to be proinflammatory (6). For hepatocyte mtDNA to activate TLR9, it would require release from hepatocytes and entry into TLR9-containing endosomes of cells capable of mounting an inflammatory response. Plasma extracellular mtDNA levels increase in tissue injuries in mice and humans including toxic liver injury (8). We tested whether a 12-week HFD model of NASH resulted in an increase in plasma mtDNA. There was an increase in total DNA and mtDNA, but not nDNA in plasma from HFD-fed compared with CD-fed mice (Supplemental Figure 1, A–C; supplemental material available online with this article; doi:10.1172/JCI83885DS1). Plasma from HFD-fed mice gave a small but significantly greater signal than did plasma from CD-fed mice from a TLR9 reporter cell line (Supplemental Figure 1D). To confirm whether these changes were present in humans, plasma from 3 previously well-characterized groups was analyzed: group 1 consisted of lean subjects without...
Liver disease and normal alanine transaminase (ALT) levels; group 2 consisted of obese subjects with normal serum ALT levels; and group 3 consisted of obese subjects with elevated ALT levels (Table 1 and ref. 9). We used ALT as a marker of ongoing liver injury. The human data mirrored the mouse HFD model data, with group 3 subjects having increased plasma levels of total DNA and mtDNA, but not nDNA compared with groups 1 and 2 (Figure 1, C–E). In addition, plasma from group 3 subjects produced a stronger signal, while the same preparations did not contain oxDNA by detecting 8-OH-deoxyguanosine (8-OH-dG) and found that there was a significant increase in oxDNA in MPs in plasma from obese subjects with elevated serum ALT levels (Figure 1K).

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TLR9 ligands can preferentially activate 2 downstream signaling pathways, resulting in differing degrees of production of NF-κB-dependent proinflammatory cytokines and IRF7-dependent type 1 IFN (13). The IRF7-dependent pathway can upregulate the IL-1 receptor antagonist (IL-1RA), which has antiinflammatory effects in the liver (14). To test whether mtDNA from hepatocytes of HFD-fed mice can activate type 1 IFN and IL-1RA, primary murine macrophages were stimulated with nDNA and mtDNA from hepatocytes of HFD-fed and CD-fed mice, and mRNA levels of Ifna, Ifnb, and Il1ra were quantified (Supplemental Figure 2, A–C). In contrast to the upregulation of TNF-α, hepatocyte mtDNA from HFD-fed mice, when compared with mtDNA from CD-fed mice, resulted in no change in Ifna, Ifnb, or Il1ra levels (15). This demonstrates that mtDNA from hepatocytes of HFD-fed mice results in the selective upregulation of proinflammatory cytokines, but not type 1 IFN.

Total body TLR9 deletion results in loss of NASH in experimental models, but the cellular requirement for TLR9 is not known (5). Liver macrophages, which include resident KCs and infiltrating monocytes, are the main proinflammatory cell populations in NASH. We tested the role of TLR9 signaling in these cells by generating mice in which TLR9 was removed from macrophages, and found that there was a significant increase in oxDNA in MPs in plasma from obese subjects with elevated serum ALT levels (Figure 1K). Interestingly, there was no increase in the percentage of free mitochondria staining for oxDNA, and the cellular origin of these free mitochondria is yet to be determined. The hepatocyte origin of plasma MPs containing mitochondria with increased oxDNA is consistent with the findings of increased oxidation of hepatocyte DNA in NASH (6). Finally, we confirmed that MPs were responsible for the ability of plasma to activate a TLR9 reporter cell line, as the majority of this activity was lost in MP-free plasma (Figure 1L).
in NASH, NF-κB activation was examined in KCs in vivo using an NF-κB reporter mouse and gating on CD11b F4/80 double-positive cells. The HFD induced upregulation of the NF-κB reporter in CD11b F4/80 double-positive cells (Supplemental Figure 3E), and KCs from WT mice on a HFD had greater proinflammatory responsiveness to a TLR9 ligand (Supplemental Figure 3, F–H).

The demonstration of high plasma TLR9 ligand activity and a requirement for TLR9 in the development of HFD-induced NASH have immediate therapeutic implications. TLR9 antagonists have excellent safety profiles and represent a novel therapeutic strategy for NASH. We tested whether the TLR7/9 antagonist IRS954 could block the ability of hepatocyte mtDNA from HFD-fed mice to activate proinflammatory cytokines in primary macrophages.

WT mice were placed on a HFD and concurrently administered mation, and elevated transaminases, and these were all significantly less severe in Tlr9-KO and Lysm-Cre Tlr9fl/fl mice (Figure 2, A–C). Tlr9-KO and Lysm-Cre Tlr9fl/fl mice also had reduced upregulation of total liver inflammatory cytokine gene expression while on a HFD (Figure 2D) and no difference in food intake (Supplemental Figure 3D). The reduction in hepatocyte steatosis in Lysm-Cre Tlr9fl/fl mice is interesting and was also found in an earlier model of methionine choline–deficient (MCD) diet steatosis in Tlr9–/– mice (5). The ability of IL-1β to induce hepatocyte steatosis suggests a direct role of proinflammatory cytokines. As lysozymes are expressed by neutrophils, monocytes, and tissue macrophages, this approach is not exclusive to liver macrophages and KCs but provides significant support for their involvement.

To further confirm that proinflammatory KC activation occurs in NASH, NF-κB activation was examined in KCs in vivo using an NF-κB reporter mouse and gating on CD11b F4/80 double-positive cells. The HFD induced upregulation of the NF-κB reporter in CD11b F4/80 double-positive cells (Supplemental Figure 3E), and KCs from WT mice on a HFD had greater proinflammatory responsiveness to a TLR9 ligand (Supplemental Figure 3, F–H).

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HMGB1, which can enhance TLR9 signaling by DNA, and additional damage-associated molecular patterns (DAMPs), such as formyl peptides (16, 17). The systemic distribution of hepatocyte-derived mitochondria with the ability to activate TLR9 may have consequences in addition to hepatic inflammation. A chronic, low-level increase in plasma TLR9 ligand activity can be expected to activate TLR9 systemically, and this is consistent with the low-level systemic inflammation in NASH that is found in many organs including white adipose tissues and its loss in the absence of TLR9 (5). This leads to the intriguing possibility that hepatocyte-derived mtDNA is responsible for activation of macrophage populations in extrahepatic sites.

**Methods**

**Statistics.** Differences between 2 groups were compared with a Mann-Whitney U test using GraphPad Prism 6 software (GraphPad Software). A P value of less than 0.05 was considered significant. See the Supplemental Methods for additional details.

**Study approval.** This study was approved by the Yale University Animal Care and Use Committee and the Human Investigation Committee.

**Author contributions**

IGM, NS, YC, RH, and XO performed experimental work, data analysis, and reviewed the manuscript. RLC, AC, and MJS were responsible for experimental design, data analysis, and review of the manuscript. SC handled clinical management and phenotypic characterization of subjects. IGM and WZM were responsible for experimental design, data analysis, and manuscript preparation.
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BRIEF REPORT

Brief report

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Figure 3. Weekly administration of a TLR9 antagonist protects against NASH. Soluble mtDNA (100 ng/ml) from hepatocytes of CD- and HFD-fed mice was added to KCs in the presence and absence of IRS954 (IRS) for 8 hours, and the upregulation of pro-Il1b and Tnfa message was quantified (A and B). In a HFD model of NASH, weekly s.c. administration of the TLR9 antagonist IRS954 (5 mg/kg, n = 5) resulted in reduced steatosis, ballooning, and inflammation (C and D), reduced ALT (E), and reduced upregulation of inflammatory cytokines in the liver (F) compared with mice fed a HFD without injection of IRS954 (n = 5). Original magnification, ×100. Data represent the mean ± SEM. *P < 0.05 and **P < 0.01, by Mann-Whitney U test.

10. Boudreau LH, et al. Platelets release mitochondria serving as substrate for bactericidal group...


