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# Macrophage CGI-58 Deficiency Activates ROS-Inflammasome Pathway to Promote Insulin Resistance in Mice

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## SUMMARY

Overnutrition activates a proinflammatory program in macrophages to induce insulin resistance (IR), but its molecular mechanisms remain incompletely understood. Here, we show that saturated fatty acid and lipopolysaccharide, two factors implicated in high-fat diet (HFD)-induced IR, suppress macrophage CGI-58 expression. Macrophage-specific CGI-58 knockout (MaKO) in mice aggravates HFD-induced glucose intolerance and IR, which is associated with augmented systemic/tissue inflammation and proinflammatory activation of adipose tissue macrophages. CGI-58-deficient macrophages exhibit mitochondrial dysfunction due to defective peroxisome proliferator-activated receptor (PPAR) $\gamma$  signaling. Consequently, they overproduce reactive oxygen species (ROS) to potentiate secretion of proinflammatory cytokines by activating NLRP3 inflammasome. Anti-ROS treatment or NLRP3 silencing prevents CGI-58-deficient macrophages from oversecreting proinflammatory cytokines and from inducing proinflammatory signaling and IR in the cocultured fat slices. Anti-ROS treatment also prevents exacerbation of inflammation and IR in HFD-fed MaKO mice. Our data thus establish CGI-58 as a suppressor of overnutrition-induced NLRP3 inflammasome activation in macrophages.

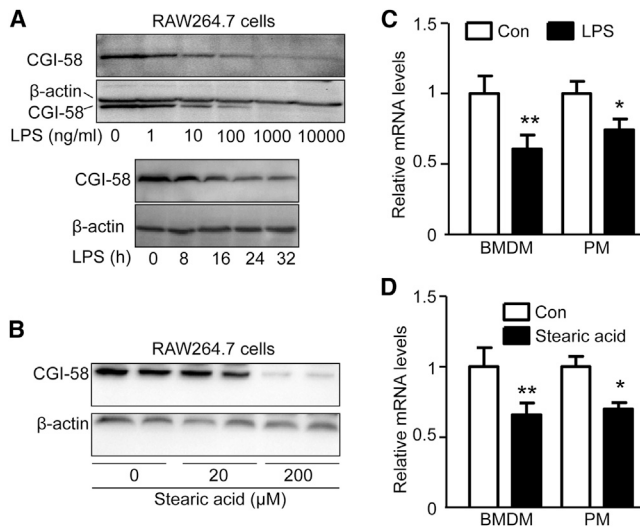
## INTRODUCTION

Obesity and diabetes are often associated with a state of low-grade chronic inflammation and insulin resistance (IR) (Hotami-

sligil, 2006; Kahn et al., 2006). Although the etiology of this chronic inflammation is likely multifactorial, increased adipose tissue infiltration of inflammatory immune cells, such as proinflammatory (M1-like) macrophages (Weisberg et al., 2003; Xu et al., 2003), is believed to be the primary source of many inflammatory cytokines. However, the molecular mediators promoting the proinflammatory property of adipose tissue macrophages remain elusive (Chawla et al., 2011).

Inflammasome activation has recently emerged as critical players in mediating immune and metabolic disorders, including obesity and IR (Martinon et al., 2009; Rathinam et al., 2012; Stienstra et al., 2011, 2012; Strowig et al., 2012; Vandanmagsar et al., 2011; Wen et al., 2012). Inflammasomes are a group of protein complexes that are formed in the cytoplasm of cells in response to pathogen-associated molecular patterns and host-derived danger-associated molecular patterns. Activation of these inflammasomes results in proteolytic cleavage of procaspase-1 to produce mature caspase-1, which cleaves pro-interleukin (IL)-1 $\beta$  and pro-IL-18, leading to secretion of potent proinflammatory cytokines IL-1 $\beta$  and IL-18. It was hypothesized that two or more signals are required for full activation of inflammasomes (the two signal hypothesis) (Gong et al., 2010; Hornung et al., 2009; Joosten et al., 2010; Stienstra et al., 2012; Wen et al., 2011). Transcription and translation of pro-IL-1 $\beta$  and pro-IL-18 are often induced by priming cells with proinflammatory stimuli, such as lipopolysaccharide (LPS), to provide the first (baseline) signal.

The nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome is of particular interest because it can sense nonmicrobial danger-associated molecular patterns such as uric acid crystals (Martinon et al., 2006) and extracellular ATP (Mariathasan et al., 2006). A recent study showed that saturated free fatty acids (FFAs) also activate the NLRP3 inflammasome to impair insulin signaling (Wen et al., 2012). Although these fatty acids may dampen insulin



**Figure 1. LPS and Saturated Fatty Acid Suppress CGI-58 Expression in Macrophages**

(A) Western blots of CGI-58 protein in RAW264.7 macrophages treated with different doses of LPS or 100 ng/ml of LPS at different time points. (B) Western blots of CGI-58 protein in RAW264.7 macrophages treated with different doses of stearic acid for 24 hr. (C and D) Relative mRNA levels of CGI-58 in overnight serum-starved BMDMs and thioglycollate-elicited PMs treated with LPS (100 ng/ml, 4 hr) (C) or stearic acid (18:0) (200  $\mu$ M, 24 hr) (D). (n = 4/group). Each error bar represents SEM.

signaling by engaging toll-like receptors (TLRs) to induce NF- $\kappa$ B-dependent production of proinflammatory cytokines such as IL-6 and tumor necrosis factor (TNF)- $\alpha$  (Shi et al., 2006), the NLRP3 inflammasome represents a novel molecular link between saturated FFAs and chronic inflammation. However, the upstream metabolic modulators of NLRP3 inflammasome activation are incompletely understood.

Reactive oxygen species (ROS) are key mediators of caspase-1 and NLRP3 inflammasome activation (Cruz et al., 2007; Dostert et al., 2008; Nakahira et al., 2011; Zhou et al., 2011). It was shown that mitochondrial dysfunction activates the NLRP3 inflammasome by causing ROS accumulation (Wen et al., 2011). Nuclear receptors peroxisome proliferator-activated receptors (PPARs) play critical roles in mitochondrial biogenesis and function (Hock and Kralli, 2009). Interestingly, PPAR $\gamma$ , PPAR $\delta$ , and PPAR $\gamma$  coactivator-1 $\beta$  (PGC-1 $\beta$ ) were shown to promote macrophage's anti-inflammatory properties to increase insulin sensitivity (Hevener et al., 2007; Kang et al., 2008; Odegaard et al., 2007, 2008; Stienstra et al., 2008; Vats et al., 2006). Although these studies directly link PPAR-sustained mitochondrial functions to macrophage's inflammatory state, the molecular mediators remain largely unknown. It was also unknown whether PPAR activities are directly linked to inflammasome activation.

It was recently reported that FFAs derived from intracellular hydrolysis mediated by Adipose Triglyceride Lipase (ATGL) activate PPARs in the heart (Haemmerle et al., 2011), suggesting an important role of intracellular fat hydrolysis in maintaining mitochondrial functions. The full activity of ATGL requires a coactivator called Comparative Gene Identification-58 (CGI-58) (Lass

et al., 2006). CGI-58 is a lipid droplet-associated protein (Liu et al., 2004; Subramanian et al., 2004; Yamaguchi et al., 2004) whose mutations in human cause Chanarin-Dorfman syndrome (CDS), a rare autosomal recessive neutral lipid storage disease characterized by ichthyosis (thickened dry skin) and accumulation of triglyceride-rich lipid droplets in most tissues and cell types, but fat (Lefevre et al., 2001). The role of CGI-58 in mediating intracellular fat hydrolysis is well established (Brown et al., 2007, 2010; Granneman et al., 2009; Lass et al., 2006; Radner et al., 2010; Zierler et al., 2013) but has never been linked to inflammasome activation.

All of the aforementioned studies collectively led us to hypothesize that CGI-58-mediated intracellular fat hydrolysis may be linked to the NLRP3 inflammasome activation via the FFA-PPAR-Mitochondria-ROS pathway. We further hypothesized that macrophage CGI-58 deficiency may exacerbate high-fat diet (HFD)-induced IR by activating the proinflammatory phenotype of tissue macrophages. To test our hypotheses, we selectively inactivated CGI-58 in macrophages of mice and examined the role of macrophage CGI-58 in inflammasome activation and the pathogenesis of HFD-induced inflammation and IR.

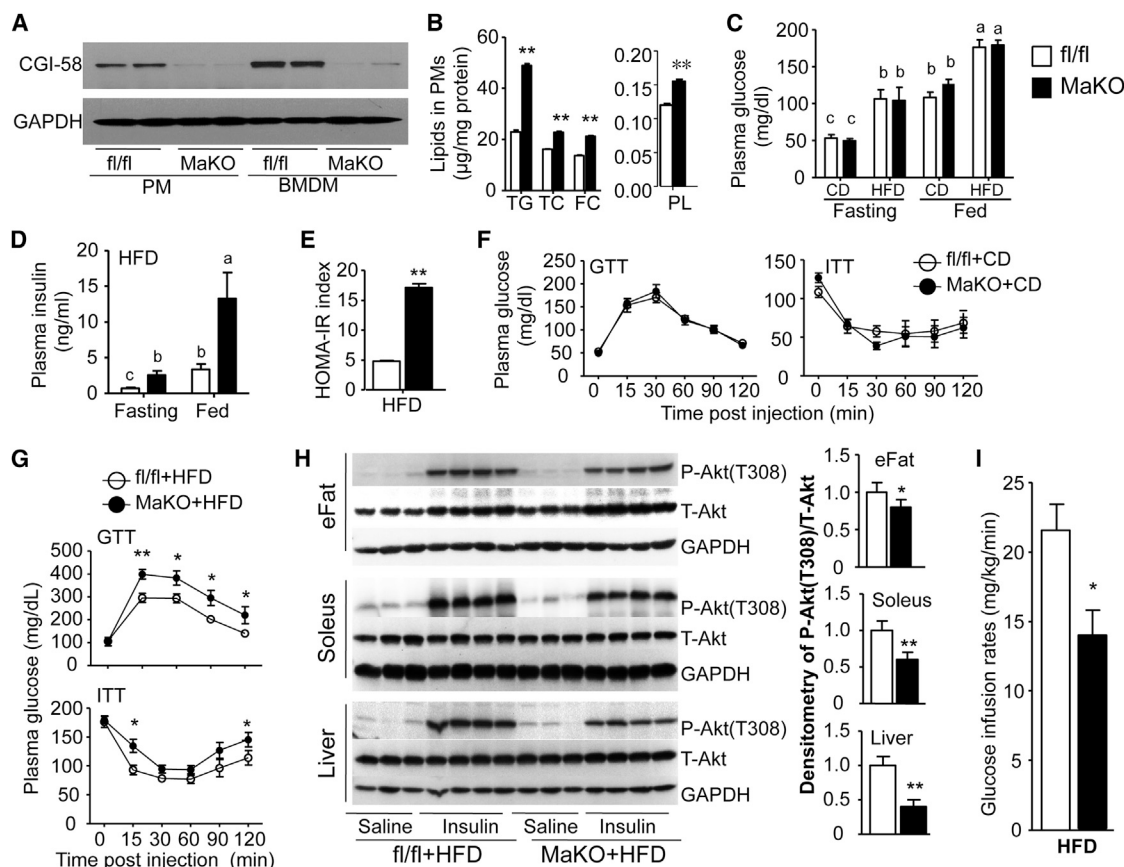
## RESULTS

### CGI-58 Expression Is Downregulated by LPS and Saturated Fatty Acid

It is currently unknown whether factors related to overnutrition-induced IR influence CGI-58 expression in macrophages. To establish the pathophysiological significance of macrophage CGI-58 in the development of IR, we treated macrophages with LPS and saturated fatty acid (stearic acid, 18:0), two factors known to be associated with overnutrition-induced obesity, IR, and type 2 diabetes (Boden et al., 1994; Kahn et al., 2006; Shi et al., 2006). LPS treatment reduced CGI-58 protein expression in a dose- and time-dependent manner in RAW264.7 murine macrophages (Figure 1A). Stearic acid treatment also reduced CGI-58 protein levels in RAW264.7 cells (Figure 1B). In addition, LPS and stearic acid both significantly reduced CGI-58 mRNA levels in bone-marrow-derived macrophages (BMDMs) and thioglycollate-elicited peritoneal macrophages (PMs) isolated from C57BL/6J mice (Figures 1C and 1D). These results indicate that macrophage CGI-58 expression is directly regulated by IR-inducing factors associated with overnutrition.

### Macrophage CGI-58 Deficiency Aggravates HFD-Induced IR

It was unknown whether macrophage CGI-58 regulates overnutrition-induced IR. To address this question, we selectively inactivated CGI-58 expression in macrophages of mice using the Cre-LoxP system. Macrophage-specific deletion of CGI-58 expression was confirmed through immunoblotting of CGI-58 protein levels in BMDMs, PMs, and multiple tissues of macrophage CGI-58-specific knockout (MaKO) mice and homozygous floxed (fl/fl) littermates (Figures 2A and S1A). The PMs from MaKO mice appeared heterogeneous in size and show a marked increase in cytosolic lipid droplets of varying sizes (Figures S1B and S1C). They had increased cellular contents of triglycerides, total cholesterol, free cholesterol, and phospholipids (Figure 2B).



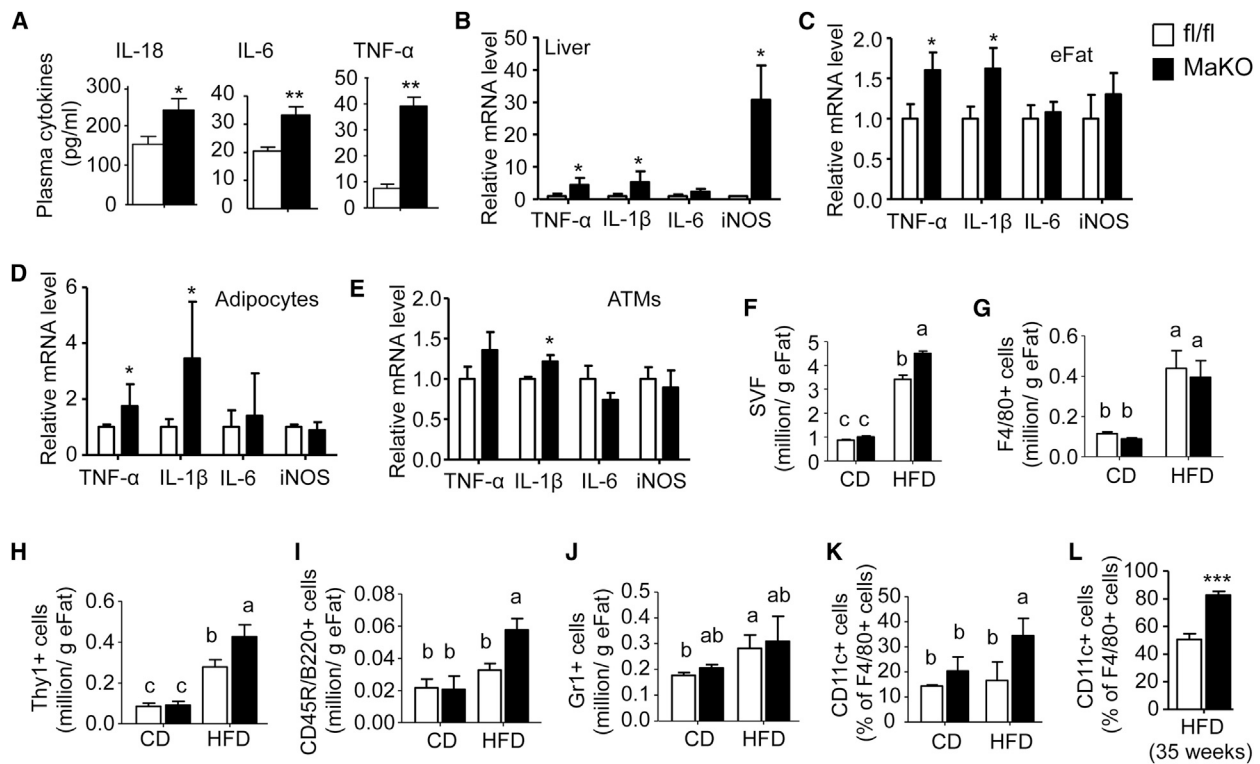
**Figure 2. Macrophage CGI-58 Deficiency Aggravates HFD-Induced IR**

(A) Western blots of CGI-58 protein in 100  $\mu$ g lysates of PMs and BMDMs from 8-week-old mice on chow diet. GAPDH as an internal control. (B) Contents of triglyceride (TG), total cholesterol (TC), free cholesterol (FC), and phospholipid (PL) in PMs from male mice on HFD for 16 weeks (n = 5). (C) Fasting (12 hr) and fed plasma glucose levels of male mice on chow diet (CD) or HFD for 16 weeks (n = 8–10). (D) Fasting (12 hr) and fed plasma insulin levels of male mice on HFD for 17 weeks. Values not sharing a common superscript letter differ significantly (n = 8–10). (E) HOMA-IR index of male mice calculated from data in (C) and (D). (F) Glucose tolerance test (GTT) and insulin tolerance test (ITT) on 20- to 21-week-old male mice on chow diet (CD) (n = 7–8). (G) Glucose tolerance test (GTT) and insulin tolerance test (ITT) on male mice on HFD for 16 weeks (n = 7–9). (H) Acute insulin signaling in epididymal fat (eFat), soleus, and liver of male mice on HFD for 16 weeks. (I) Glucose infusion rates measured by hyperinsulinemic-euglycemic clamp studies in male mice on HFD for 35 weeks (n = 5). Each error bar represents SEM.

The majority of triglyceride molecular species as well as fatty acid species in triglycerides were increased (Tables S1 and S2), and levels of total ceramides and phosphatidylinositols were decreased in these cells. There were no significant differences in PM contents of total acyl-carnitines, cardiolipins, lysophosphatidylcholines, phosphatidic acids, phosphatidylglycerols, phosphatidylserines, sphingomyelins, phosphatidylcholines, and phosphatidylethanolamines between the two genotypes, although some molecular species of these lipids showed significant changes (Table S3). These results demonstrate that CGI-58 plays a crucial role in maintaining homeostasis of major cellular lipids in macrophages. Despite this, macrophage CGI-58 deficiency had minimal impact on cholesterol, total triglycerides, and phospholipids in the plasma, liver, and fat (Table S4).

To determine if macrophage CGI-58 deficiency alters insulin sensitivity, we challenged mice with a HFD. CGI-58 deficiency

did not affect weight gain during 18 weeks of diet feeding, starting at 4 weeks of age. As expected, HFD relative to chow diet induced a significant increase in weight gain (Figure S1D). Although fasting or fed plasma glucose concentrations did not differ between the two groups regardless of diets (Figure 2C), MaKO mice on HFD showed significant increases in fasting and fed plasma insulin (Figure 2D) as well as HOMA-IR index (Figure 2E), indicative of augmented IR. To further assess systemic insulin sensitivity, we performed glucose and insulin tolerance tests. Glucose tolerance and insulin sensitivity were unaltered in chow-fed mice (Figure 2F) but impaired in HFD-fed MaKO mice (Figure 2G). When acute insulin signaling was examined in mice on HFD for 18 weeks, insulin-stimulated AKT (Thr308) phosphorylation was significantly attenuated by 20%, 35%, and 58% in epididymal fat, soleus muscle, and liver, respectively, in MaKO mice relative to controls



**Figure 3. Macrophage CGI-58 Deficiency Aggravates HFD-Induced Inflammation**

(A) Plasma concentrations of cytokines in male mice on HFD for 16 weeks (n = 5).  
 (B) Liver inflammatory cytokine mRNA levels (n = 4).  
 (C) Epididymal fat (eFat) inflammatory cytokine mRNA levels (n = 4).  
 (D) Inflammatory cytokine mRNA levels in epididymal fat-derived adipocytes (n = 4).  
 (E) Inflammatory cytokine mRNA levels in epididymal fat-derived macrophages (ATMs) (n = 4).  
 (F) Total cell number of stromal-vascular fraction (SVF) in epididymal fat (eFat) pads isolated from age-matched male mice on chow diet (CD) or HFD for 16 weeks (n = 8–12).  
 (G) F4/80<sup>+</sup> macrophage number in epididymal fat (eFat) (n = 8–12).  
 (H) Thy1<sup>+</sup> T cell number in epididymal fat (eFat) (n = 8–12).  
 (I) CD45R/B220<sup>+</sup> B cell number in epididymal fat (eFat) (n = 8–12).  
 (J) Gr1<sup>+</sup> neutrophil number in epididymal fat (eFat) (n = 8–12).  
 (K) Percentage of CD11c<sup>+</sup> cells in F4/80<sup>+</sup> cell population in epididymal fat from age-matched male mice on chow diet (CD) or HFD for 16 weeks (n = 8–12).  
 (L) Percentage of CD11c<sup>+</sup> cells in F4/80<sup>+</sup> cell population in epididymal fat from age-matched male mice on HFD for 35 weeks (n = 5).  
 Each error bar represents SEM.

(Figure 2H). Furthermore, hyperinsulinemic-euglycemic clamp studies showed that the glucose infusion rate was significantly lower in MaKO mice than f/f mice on HFD for 35 weeks (Figure 2I). Taken together, these results demonstrate that loss of CGI-58 in macrophages aggravates HFD-induced IR in mice.

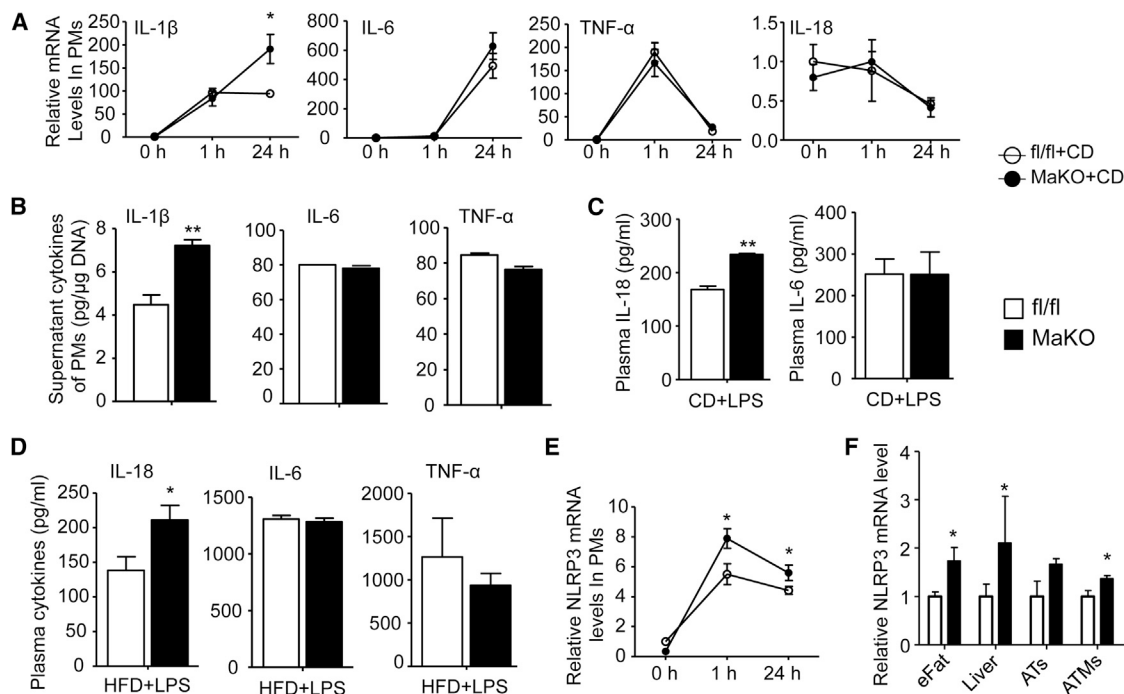
### Macrophage CGI-58 Deficiency Aggravates HFD-Induced Inflammation

To test whether macrophage CGI-58-deficiency-induced IR is associated with augmented inflammation, we measured plasma and tissue levels of proinflammatory cytokines. Plasma concentrations of IL-18, IL-6, and TNF- $\alpha$  were significantly higher in MaKO mice than controls (Figure 3A). Hematoxylin and eosin (H&E) staining revealed increased infiltration of inflammatory cells in liver (data not shown) and epididymal fat (Figure S2A) of HFD-fed MaKO mice. Consistently, MaKO

mice showed a significant increase in mRNA abundance of TNF- $\alpha$ , IL-1 $\beta$ , and inducible nitric oxide synthase (iNOS) in liver (Figure 3B) and TNF- $\alpha$  and IL-1 $\beta$  in fat (Figure 3C). Similar changes were observed for these mRNAs in epididymal fat-derived adipocytes (Figure 3D) and macrophages (ATMs) (Figure 3E). These results indicate that macrophage CGI-58 deficiency exacerbates HFD-induced tissue inflammation in mice.

### Increased Infiltration of M1-like Macrophages, T Cells, and B Cells in the Epididymal Fat of HFD-Fed MaKO Mice

To determine whether the augmented inflammation in the adipose tissue of MaKO mice is linked to proinflammatory activation of adipose tissue macrophages, we isolated the stromal-vascular fraction (SVF) of the epididymal fat pads and performed fluorescence-activated cell sorting (FACS) analysis (Figures S2B



**Figure 4. Selective Increases of IL-1 Family Cytokines in LPS-Primed CGI-58-Deficient PMs and Mice Correlate with Increased NLRP3 Expression**

(A) Cytokine mRNA levels in PMs from 12-week-old chow-fed male mice treated with LPS (100 ng/ml) (n = 4).

(B) Proinflammatory cytokine concentrations in the supernatant (conditional culture medium) of PMs treated with LPS for 24 hr (n = 4).

(C) Plasma cytokine concentrations in 20-week-old chow-fed male mice. Mice were fasted for 4 hr and blood samples were collected 24 hr postintra-peritoneal injection of LPS (50 ng/g body weight) (n = 5).

(D) Plasma cytokine concentrations in male mice on HFD for 8 weeks. Mice were fasted for 4 hr and plasma samples were collected 24 hr after intraperitoneal injection of LPS (50 ng/g body weight) (n = 5).

(E) NLRP3 mRNA levels in LPS (100 ng/ml)-treated PMs from 12-week-old chow-fed male mice (n = 4).

(F) NLRP3 mRNA levels in epididymal fat (eFat), liver, eFat-derived adipocytes (ATs), and macrophages (ATMs) from male mice on HFD for 16 weeks (n = 3–5).

Each error bar represents SEM.

and S2C). On chow diet, no differences were observed in the total number of SVF cells (Figure 3F), F4/80<sup>+</sup> cells (macrophages) (Figure 3G), Thy1<sup>+</sup> cells (T cells) (Figure 3H), CD45R/B220<sup>+</sup> cells (B cells) (Figure 3I), and Gr1<sup>+</sup> cells (neutrophils) (Figure 3J) between the two genotypes. On HFD, the total number of SVF cells, Thy1<sup>+</sup> cells, and CD45R/B220<sup>+</sup> cells were significantly higher in MaKO mice than controls, whereas the total number of F4/80<sup>+</sup> cells and Gr1<sup>+</sup> cells remained unaltered. The percentage of CD11c<sup>+</sup> cells in the F4/80<sup>+</sup> population was significantly increased in MaKO mice on HFD for 16 weeks (Figure 3K) and for 35 weeks (Figure 3L), indicative of increased proinflammatory macrophages in the adipose tissue.

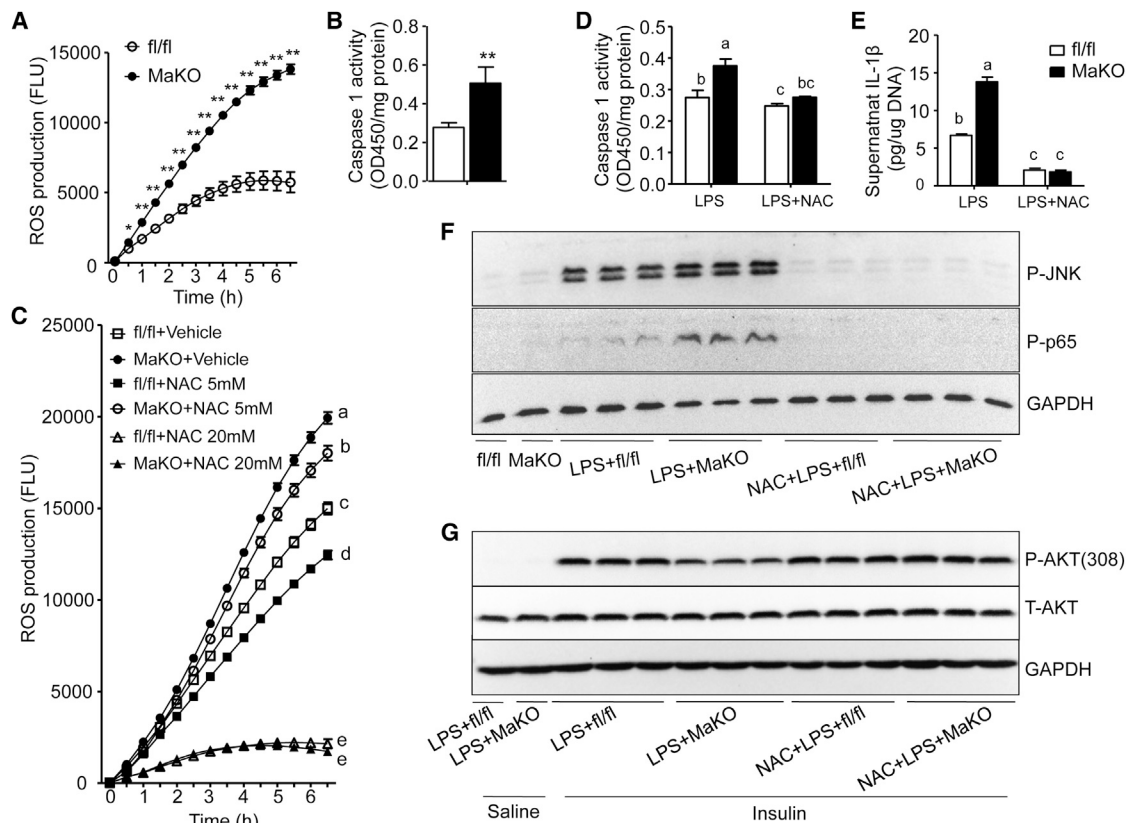
We also analyzed splenocytes and blood immune cells by FACS analysis. No genotype-related differences were seen for the percentages of F4/80<sup>+</sup> cells, Thy1<sup>+</sup> cells, and Gr1<sup>+</sup> cells in total splenocytes and total white blood cells, or for the percentage of CD11c<sup>+</sup> cells in the F4/80<sup>+</sup> cell population in these cell preparations, regardless of diets (Figures S2D–S2M). Intriguingly, HFD feeding significantly reduced the percentage of CD45R/B220<sup>+</sup> cells in total splenocytes, regardless of genotypes (Figure S2F). The percentage of CD45R/B220<sup>+</sup> cells was

significantly increased in total white blood cells of MaKO mice (Figure S2K), although this increase did not reach statistical significance for total splenocytes (Figure S2F).

#### MaKO Mice Display Increased IL-1 Family Cytokines and NLRP3 Expression in Macrophages and Tissues

To explore the mechanism underlying macrophage CGI-58-deficiency-induced inflammation, we primed the isolated PMs with LPS to stimulate proinflammatory responses and monitored expression and secretion of proinflammatory cytokines. No differences were observed between the two groups in macrophage levels of mRNAs for IL-6, TNF- $\alpha$ , and IL-18 after LPS stimulation for 1 hr and for 24 hr (Figure 4A). At 24 hr, compared with fl/fl group, PMs from MaKO mice showed a specific and significant increase in IL-1 $\beta$  mRNA (Figure 4A). Consistently, these PMs secreted more IL-1 $\beta$  protein at this time point, though the secreted TNF- $\alpha$  and IL-6 remained comparable between the two groups (Figure 4B).

To determine if our in vitro observation can be recapitulated in vivo, we measured plasma proinflammatory cytokine levels in chow- and HFD-fed mice injected intraperitoneally with LPS



**Figure 5. ROS Is Critically Involved in Macrophage CGI-58 Deficiency-Associated Inflammasome Activation, Inflammation, and IR**

(A) ROS production in PMs from male mice on chow diet (n = 6).  
 (B) Caspase-1 activity in PMs from 12-week-old male mice on chow diet (n = 6).  
 (C) ROS production in PMs from 12-week-old male mice treated with NAC (20 mM) or vehicle for 24 hr (n = 6).  
 (D) Caspase-1 activity in PMs from 12-week-old male mice treated with LPS (100 ng/ml) plus NAC (20 mM) or vehicle for 24 hr (n = 4).  
 (E) Secreted IL-1 $\beta$  in the conditional medium of PMs described in (D).  
 (F) Immunoblots of phosphorylated (P-) JNK and p65 in the epididymal fat pad slices (from 12-week-old wild-type C57BL/6 mice pads) cocultured with PMs from 12-week-old male fl/fl and MaKO mice in the presence of LPS (100 ng/ml) plus NAC (20 mM) or vehicle for 24 hr.  
 (G) Immunoblots of phosphorylated-AKT (Thr308) and total AKT in the fat slices cocultured with PMs in the presence of LPS (100 ng/ml) for 24 hr, followed by insulin treatment (100 nM) for 30 min in the serum-free medium.  
 Each error bar represents SEM.

at 50 ng/g body weight. Under both dietary conditions, compared with fl/fl mice, MaKO mice displayed increased plasma concentrations of IL-18, but not IL-6 and TNF- $\alpha$  (Figures 4C and 4D) 24 hr post LPS injection. IL-1 $\beta$  was undetectable at this time point.

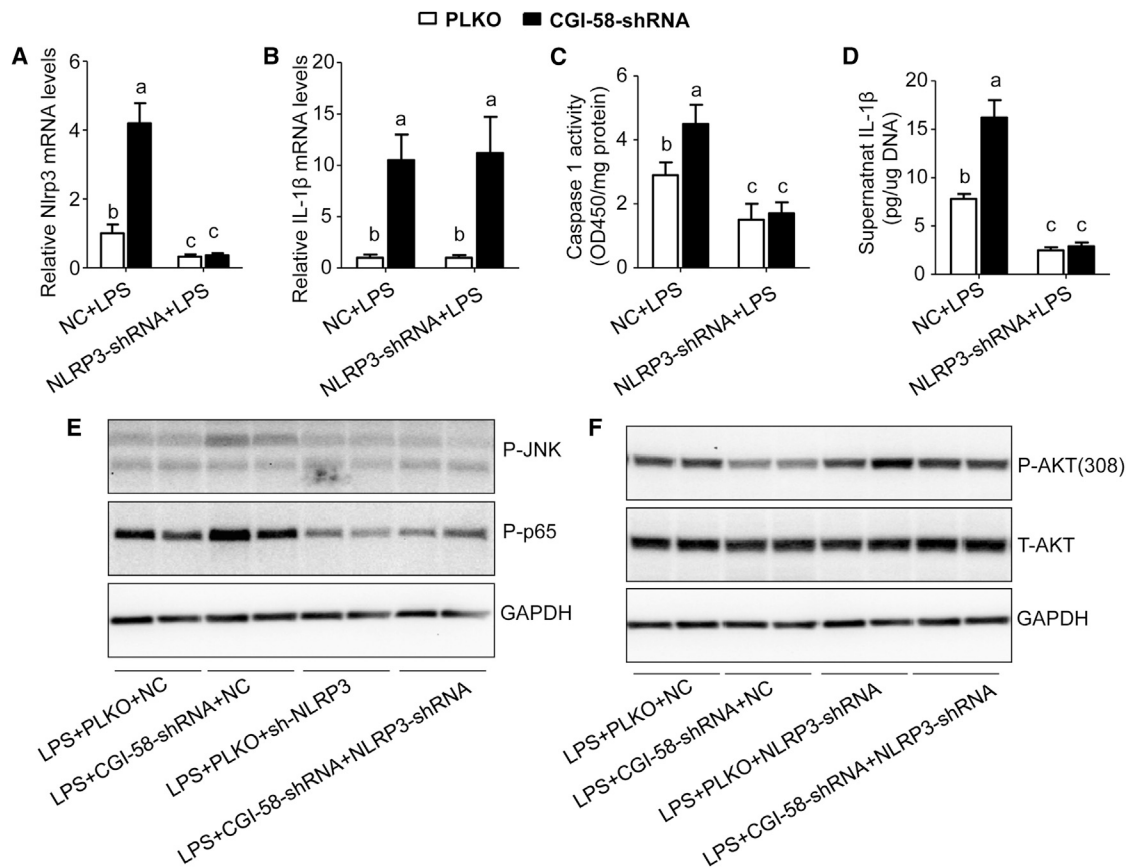
To examine whether the selective induction of IL-1 family cytokines (IL-1 $\beta$  or IL-18) in CGI-58-deficient macrophages or mice is associated with increased expression of NLRP3, we measured NLRP3 mRNA levels in macrophages and tissues. After LPS stimulation, MaKO-mice-derived PMs had increased NLRP3 mRNA (Figure 4E). When isolated PMs and BMDMs were treated with stearic acid (18:0) to mimic overnutrition, NLRP3 mRNA levels were significantly higher in macrophages from MaKO mice than controls (Figures S3A and S3B). On HFD, MaKO mice versus fl/fl controls had increased NLRP3 mRNA levels in epididymal fat, liver, and adipose tissue-derived macrophages (ATMs) (Figure 4F). Collectively, these results suggest that the

NLRP3 inflammasome may be activated in CGI-58-deficient macrophages.

#### CGI-58-Deficient Primary Macrophages Overproduce ROS and Display ROS-Dependent Increases in Caspase-1 Activity

Because ROS play a critical role in the activation of NLRP3 inflammasome, to determine if CGI-58-deficient macrophages accumulate ROS, we measured ROS production in PMs from MaKO and control mice. PMs from MaKO mice showed significant increases in ROS production (Figure 5A) and caspase-1 activity (Figure 5B), indicative of activation of the inflammasome pathway. Treatment with an anti-ROS agent N-acetyl-cysteine (NAC) completely prevented this ROS overproduction (Figure 5C). Additionally, in LPS-stimulated PMs, NAC treatment abolished caspase-1 activation (Figure 5D) and IL-1 $\beta$  secretion (Figure 5E) induced by CGI-58 knockout.





**Figure 6. NLRP3 Inflammasome Mediates CGI-58-Deficient Macrophages-Induced Inflammation and IR in Fat Slices**

(A) NLRP3 mRNA levels in CGI-58-silenced and PLKO control RAW264.7 cells stably transfected with NLRP3 shRNA or nontargeting control (NC) shRNA plasmids and treated with LPS (100 ng/ml) for 24 hr (n = 5).  
 (B) IL-1 $\beta$  mRNA levels in the cells described in (A).  
 (C) Caspase-1 activity in the lysates of the cells described in (A).  
 (D) IL-1 $\beta$  protein in the conditional medium of the cells described in (A).  
 (E) Immunoblots of phosphorylated (P-) JNK and p65 in the epididymal fat pad slices cocultured with different RAW264.7 cells in the presence of LPS (100 ng/ml, 24 hr).  
 (F) Immunoblots of phosphorylated (P-) AKT (Thr308) and total AKT (T-AKT) in the epididymal fat pad slices cocultured with different RAW264.7 cells RAW264.7 cells in the presence of LPS (100 ng/ml) for 24 hr, followed by medium change to serum-free medium and treatment with insulin (100 nM) for 30 min. Each error bar represents SEM.

### NAC Treatment Prevents CGI-58-Deficient PMs-Induced Inflammation and IR in Fat Slices

To examine whether CGI-58-deficient primary PMs directly induce inflammatory signaling and dampen insulin signaling in adipose tissue, we performed the coculture experiments using epididymal fat pads from C57BL/6 wild-type mice and PMs from either MaKO mice or fl/fl control mice. The coculture was primed with LPS to induce observable inflammatory signaling and to suppress the baseline insulin signaling. Clearly, PMs from MaKO mice relative controls increased the phosphorylation levels of JNK and p65 proteins in the cocultured fat slices (Figure 5F). NAC treatment prevented LPS-induced phosphorylation of JNK and p65 and abolished the phosphorylation differences of JNK and p65 between the two groups (Figure 5F). The insulin signaling was attenuated in the epididymal fat slices cocultured with PMs from MaKO mice, and this attenuation was completely

restored by NAC treatment (Figure 5G). Altogether, these ex vivo data indicate that CGI-58-deficient macrophages directly activate proinflammatory signaling and suppress insulin signaling in fat slices in a ROS-dependent manner.

### CGI-58-Deficient Murine RAW264.7 Macrophages Induce Adipose Tissue Inflammation and IR in an NLRP3-Dependent Manner

To directly determine if increased secretion of IL-1 $\beta$  in CGI-58-deficient macrophages is NLRP3 inflammasome dependent, we employed murine RAW264.7 cells as a model and silenced CGI-58 and NLRP3 expression using lentiviral small hairpin RNAs (shRNAs). Silencing of CGI-58 was confirmed by immunoblotting of CGI-58 protein (Figure S4A) and oil red O staining of lipid droplets (Figure S4B). Silencing of NLRP3 was validated by quantitative PCR (qPCR) (Figure 6A). As expected, silencing

of CGI-58 significantly increased secretion of IL-1 $\beta$  and IL-18, but not TNF- $\alpha$  and IL-6 in RAW264.7 cells primed with LPS (Figure S4C). Silencing of CGI-58 also increased NLRP3 protein expression induced by LPS (Figure S4D) or stearic acid (Figure S4E), and this increase was completely abolished after NAC treatment (Figures S4D and S4E), or the treatment with another antioxidant L-glutathione (GSH, reduced form) (Figures 4G and S4F). Additionally, CGI-58 silencing in RAW264.7 cells induced a dramatic increase in ROS production, NLRP3 mRNA expression, caspase-1 activity, and secretion of IL-1 $\beta$  and IL-18, all of which were inhibited by GSH treatment (Figures S5A–S5E). These results recapitulated our findings from PMs, demonstrating that RAW264.7 cell line is a reliable model for studying CGI-58 functions in macrophages. We then focused on the regulation of IL-1 $\beta$  expression/secretion and caspase-1 activity by NLRP3 in this cell line. Consistent with the data from primary PMs (Figure 4A), silencing of CGI-58 dramatically increased IL-1 $\beta$  mRNA levels in RAW264.7 cells primed with LPS (Figure 6B). Interestingly, the increase in IL-1 $\beta$  mRNA appeared to be NLRP3-independent because NLRP3 silencing failed to prevent this increase (Figure 6B), suggesting that CGI-58 deficiency may raise IL-1 $\beta$  mRNA levels by increasing transcription and/or mRNA stability. Nonetheless, CGI-58 silencing increased caspase-1 activity (Figure 6C) and IL-1 $\beta$  secretion (Figure 6D), and these increases were clearly NLRP3-dependent.

To determine if CGI-58-deficient macrophages augment adipose tissue inflammatory signaling in an NLRP3-dependent manner, we cocultured RAW264.7 cells with small slices of epididymal fat pads isolated from wild-type C57BL/6J mice on chow diet. Inflammatory signal transduction was induced by LPS treatment. The phosphorylation levels of JNK and p65 were higher in the fat tissues cocultured with CGI-58-silenced RAW264.7 cells than the control cells, and these changes were not observed when NLRP3 was silenced (Figure 6E), indicating that CGI-58-deficient macrophages require NLRP3 to stimulate proinflammatory signaling.

To determine whether CGI-58-deficient macrophages dampen adipose tissue insulin signaling in an NLRP3-dependent manner, we examined insulin signaling in our coculture system. As shown in Figure 6F, the insulin-stimulated phosphorylation of AKT at Thr308 was attenuated in the fat slices cocultured with CGI-58-silenced RAW264.7 cells, and this attenuation was rescued by NLRP3 silencing in these cells, demonstrating that CGI-58-deficient macrophages requires NLRP3 to suppress insulin signaling.

### ROS Overproduction in CGI-58-Deficient Macrophages Results from Mitochondrial Dysfunction Induced by Defective PPAR $\gamma$ Signaling

Mitochondria are the major source of cellular ROS (Handy and Loscalzo, 2012). Dysfunction of mitochondria has the potential to raise cellular ROS (Aflaki et al., 2011a, 2011b; Wen et al., 2011). To determine if CGI-58 deficiency in macrophages impairs mitochondrial biogenesis and function, we measured macrophage contents of DNAs for mitochondrial genes NADH dehydrogenase subunit 1 and cytochrome b, and for nuclear gene H19 by qPCR, and found that the ratio of mitochondrial DNA to nuclear DNA decreased significantly

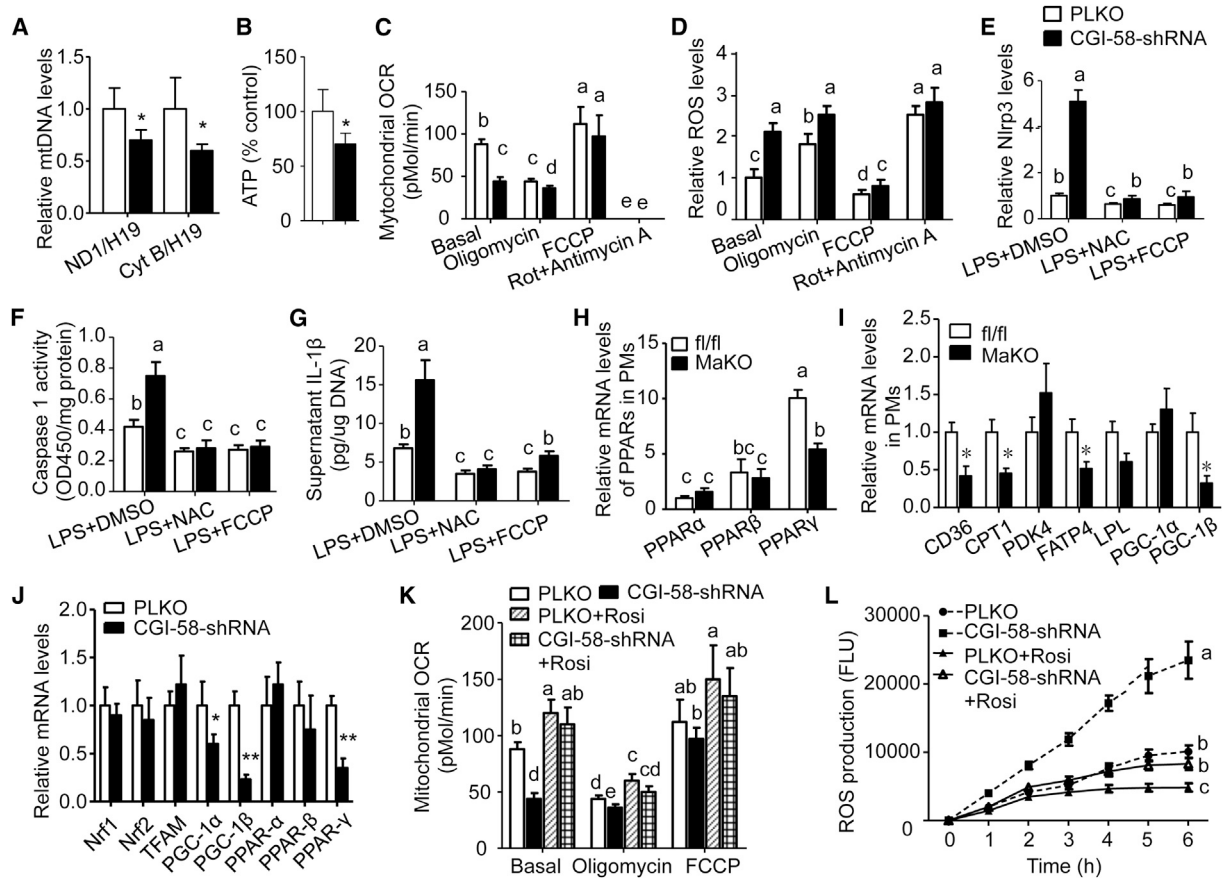
in CGI-58-silenced RAW264.7 cells (Figure 7A), indicative of reduced mitochondria. Mitochondrial ATP levels also decreased in these cells (Figure 7B). Consistently, seahorse mitochondrial function assays showed that the oxygen consumption rate (OCR) was substantially reduced in CGI-58-silenced RAW264.7 cells (Figure 7C).

To determine if manipulating mitochondrial functions will influence ROS production in CGI-58-deficient macrophages, we treated CGI-58-silenced and control RAW264.7 cells with several compounds known to alter different mitochondrial functions. As shown in Figure 7D, oligomycin, an ATP synthase inhibitor, narrowed the difference of ROS production between the two groups. Carbonyl-cyanide-*p*-trifluoro-methoxy-phenylhydrazone (FCCP), a potent uncoupler of mitochondrial oxidative phosphorylation, reduced ROS production in general and abolished the CGI-58-silencing-associated increase in ROS production. The combined inhibition of mitochondrial complex I by rotenone and complex III by antimycin A increased ROS production in general and cancelled the difference of ROS production between the two groups.

To determine if the activation of NLRP3 inflammasome depends on mitochondrial dysfunction, we treated CGI-58-silenced and control RAW264.7 cells with FCCP. This treatment, like the antioxidant NAC, abolished the CGI-58-silencing-associated increases in NLRP3 mRNA, caspase-1 activity, and IL-1 $\beta$  secretion (Figures 7E–7G), suggesting that NLRP3 inflammasome activation in CGI-58-deficient macrophages is induced by mitochondrial dysfunction.

PPARs play critical roles in mitochondrial biogenesis and function (Hock and Kralli, 2009). Cardiac deficiency of ATGL, a lipase target of CGI-58, has been reported to inhibit PPAR activity by reducing endogenous ligands for PPARs (Haemmerle et al., 2011). We speculated that PPARs might involve CGI-58-deficiency-induced mitochondrial dysfunction and ROS production. To examine this possibility, we measured the mRNA levels of PPARs and their target genes in primary PMs and found that PPAR $\gamma$  was the only isoform that was significantly altered (downregulated) in PMs from MaKO mice (Figure 7H). The mRNA for PGC-1 $\beta$ , not PGC-1 $\alpha$ , was substantially reduced in CGI-58-deficient PMs (Figure 7I). As expected, the mRNA levels of PPAR target genes such as CD36, CPT-1, FABP4, and LPL were all decreased in these PMs relative to controls. Silencing of CGI-58 in RAW264.7 cells caused similar changes in mRNA levels of PPAR $\gamma$  and PGC-1 $\beta$ , but not nuclear respiratory factor 1 (Nrf1), Nrf2, and the mammalian mitochondrial transcription factor A (TFAM) (Figure 7J). These results indicate that PPAR $\gamma$ /PGC-1 activity was inhibited in CGI-58-deficient macrophages.

To determine if PPAR $\gamma$  activation by exogenous ligands rescues CGI-58-deficiency-associated mitochondrial dysfunction and ROS overproduction, we treated CGI-58-silenced and control RAW264.7 macrophages with PPAR $\gamma$ -specific agonist rosiglitazone. We found that rosiglitazone completely restored oxygen consumption rates (Figure 7K) and inhibited ROS overproduction (Figure 7L) associated with CGI-58 silencing. These results indicate that CGI-58 deficiency induces mitochondrial dysfunction and ROS overproduction by suppressing PPAR $\gamma$  signaling.



**Figure 7. Macrophage CGI-58 Deficiency Promotes ROS Production and Inflammasome Activation by Suppressing PPAR $\gamma$ -Dependent Mitochondrial Function**

(A) DNA levels of mitochondrial (mt) genes encoding mitochondrial NADH dehydrogenase subunit-1 (ND1) and cytochrome b (Cyt. B), which were normalized to those of the nuclear gene H19 in CGI-58-silenced and control RAW264.7 cells (n = 5).  
 (B) ATP levels in RAW264.7 cells (n = 5).  
 (C) Mitochondrial oxygen consumption rates (OCRs) measured by seahorse studies (n = 5).  
 (D) Cellular ROS levels in RAW264.7 cells treated with oligomycin (2  $\mu$ g/ml), FCCP (1  $\mu$ g/ml), or rotenone-antimycin A (1  $\mu$ g/ml) for 12 hr. Measurements were done at 3 hr after 2',7'-dichlorofluorescein diacetate (DCFDA) addition (50  $\mu$ M) (n = 5).  
 (E) NLRP3 mRNA levels in RAW264.7 cells treated with LPS (100 ng/ml) for 24 hr (n = 5). In the last 12 hr of LPS treatment, NAC (20 mM), FCCP (1  $\mu$ g/ml), or vehicle DMSO was added.  
 (F) Caspase-1 activity in the lysates of a subgroup of the cells described in (E).  
 (G) IL-1 $\beta$  protein levels in the conditional medium of the cells described in (E).  
 (H) Relative mRNA levels of PPAR $\alpha$ ,  $\delta$ , and  $\gamma$  in PMs isolated from 16-week-old mice on chow diet (n = 5).  
 (I) Relative mRNA levels of PGC1- $\alpha$ , PGC1- $\beta$ , and PPAR target genes in PMs described in (H).  
 (J) Relative mRNA levels of genes related to regulation of mitochondrial function and biogenesis in RAW264.7 cells (n = 5).  
 (K) Mitochondrial oxygen consumption rates (OCRs) in RAW264.7 cells treated with rosiglitazone (Rosi) at 50 nM or vehicle DMSO for 24 hr (n = 5).  
 (L) ROS production in RAW264.7 cells treated with rosiglitazone (Rosi) at 50 nM or vehicle DMSO for 24 hr (n = 6).  
 Each error bar represents SEM.

### NAC Administration Prevents Aggravation of HFD-Induced Inflammation and IR in MaKO Mice

To determine if inhibition of ROS production rescues macrophage CGI-58-deficiency-associated inflammation and IR in vivo, we administered NAC in drinking water to our animals for 4 weeks, starting at 16 weeks of HFD feeding, and then assessed systemic insulin sensitivity and tissue inflammation. Consistent with in vitro and ex vivo data, NAC administration completely abolished the exacerbation of glucose intolerance and IR in MaKO mice (Figures S7A and S7B). In Figure 3A, we

have shown that macrophage CGI-58 deficiency raises plasma concentrations of IL-18, IL-6, and TNF- $\alpha$  in HFD-fed mice. Here, we found that NAC treatment prevented this elevation (Figure S7C). Likewise, in Figures 3B, 3C, and 4F, we have shown that hepatic and fat mRNAs for IL-1 $\beta$ , TNF- $\alpha$ , and NLRP3 were increased in MaKO mice. Here, we showed that these increases were prevented by NAC administration (Figures S7D and S7E). In addition, we did not observe any differences in mRNA levels of other inflammation-related genes examined between the two groups in the presence of NAC. Collectively, these in vivo data

demonstrate that the exacerbation of inflammation and IR in HFD-fed MaKO mice is ROS dependent.

### ATGL Silencing in RAW264.7 Cells Does Not Activate NLRP3 Inflammasome

An *in vitro* study showed that CGI-58 promotes intracellular fat breakdown by activating ATGL's triglyceride hydrolase activity (Lass et al., 2006). To determine if ATGL deficiency has similar effects on NLRP3 inflammasome, we silenced ATGL expression in RAW264.7 cells (Figure S7A). Interestingly, despite increased ROS production (Figure S7B), we did not see any changes in NLRP3 protein levels, caspase-1 activity, and secretion of TNF- $\alpha$ , IL-1 $\beta$ , and IL-18 under both unstimulated and LPS-stimulated conditions in ATGL-silenced cells (Figures S7C–S7G). The secretion of IL-6 was even reduced under LPS-stimulated state (Figure S7H). Thus, macrophage CGI-58 deficiency unlikely activates the NLRP3 inflammasome simply by inhibiting ATGL activity.

The net intracellular fat lipolysis is mediated not only by cytosolic ATGL, but also via lysosome-based lipophagy, a lipid-specific macroautophagy (Singh et al., 2009). We found that the protein level of microtubule-associated protein 1 light chain 3 $\beta$ -II (LC3b-II) was decreased in CGI-58-deficient RAW264.7 cells (Figure S7I). LC3b-II is the lipidated form of LC3b-I and a reliable marker of cell's autophagy state because this lipidated form correlates well with the number of cell's autophagosomes (Klionsky et al., 2012). This observation suggests that future studies are needed to clarify whether CGI-58 mediates lipophagy.

## DISCUSSION

In this study, using a newly created mouse model, we demonstrate that CGI-58, a lipolytic factor, is an endogenous suppressor of the NLRP3 inflammasome activity in macrophages. We show that macrophage CGI-58 deficiency activates NLRP3 inflammasome by inducing ROS accumulation through suppression of PPAR $\gamma$ -dependent mitochondrial function. A pathological consequence of macrophage CGI-58 deficiency is the exacerbation of HFD-induced inflammation and IR in mice.

Although CGI-58 may promote intracellular fat breakdown by activating ATGL (Lass et al., 2006), mutations in these two proteins cause some distinct phenotypes in humans and mice (Fischer et al., 2007; Guo et al., 2013; Haemmerle et al., 2006; Radner et al., 2010; Wu et al., 2011). When ATGL is silenced in RAW264.7 cells (Figure S7), there is no activation of the NLRP3 inflammasome, despite increased ROS production. Therefore, CGI-58 must have ATGL-independent functions, at least in some tissues and cell types. Intracellular fat lipolysis is mediated not only by ATGL, but also via a lipid-specific macroautophagy (lipophagy) (Singh et al., 2009). We found that a macroautophagy marker LC3b-II is decreased in CGI-58-deficient RAW264.7 cells (Figure S7I). Thus, it is tempting to speculate that CGI-58 deficiency may suppress intracellular fat lipolysis by inhibiting both ATGL and lipophagy.

The ATGL-independent functions of CGI-58 may explain why ATGL-deficient macrophages display an anti-inflammatory M2-like phenotype despite ROS overproduction (Aflaki et al.,

2011a, 2011b), whereas CGI-58-deficient macrophages show proinflammatory M1-like phenotype. Perhaps, ROS-dependent activation of the NLRP3 inflammasome requires additional signals, and these additional signals do not exist in ATGL-deficient macrophages. Alternatively, some of ROS produced from ATGL-deficient macrophages may be anti-inflammatory, and the ROS generated by CGI-58-deficient macrophages may be proinflammatory. It was reported that the ROS produced from the NADPH system has anti-inflammatory effects (van de Veerdonk et al., 2010). Although it is currently unknown if ATGL deficiency stimulates ROS production from NADPH system in macrophages, we did notice that ATGL relative to CGI-58 deficiency caused a substantial higher level of ROS production in RAW264.7 macrophages (Figure S7B versus Figures 7L and S5A). It is possible that CGI-58-deficient macrophages, and perhaps hepatocytes (Guo et al., 2013), lack the activation of anti-inflammatory ROS production, thus making these cells proinflammatory as seen in the patients with chronic granulomatous disease (van de Veerdonk et al., 2010).

Our data (Figure 7H) are consistent with that PPAR $\gamma$  and PPAR $\delta$  are abundantly expressed in mouse and human macrophages (Chawla, 2010). PMs from MaKO mice express substantially lower levels of PPAR $\gamma$ , PGC-1 $\beta$ , and their target genes. It is currently unclear how macrophage CGI-58 deficiency downregulates PPAR $\gamma$ /PGC-1 signaling. Cardiac deletion of ATGL leads to defective PPAR $\alpha$ /PGC-1 signaling likely via limiting availability of FFAs as endogenous ligands for PPAR $\alpha$  (Haemmerle et al., 2011). Likewise, CGI-58 deficiency may impair PPAR $\gamma$  signaling by limiting the release of FFAs from ATGL pathway and perhaps from lipophagy pathway. Nonetheless, cytosolic lipid droplets accumulated in CGI-58-deficient macrophages may sequester PPAR ligands FFAs, thereby preventing them from entering the PPAR-activating pool/compartments to fuel mitochondrial functions. Concomitantly, dysfunctional mitochondria increase. On the face of potential defects of autophagy in CGI-58-deficient macrophages (Figure S7I), the clearance of these dysfunctional mitochondria via mitophagy may be attenuated, therefore increasing ROS production. PPAR $\gamma$ , PPAR $\delta$ , and PGC-1 $\beta$  were shown to promote macrophages' anti-inflammatory properties to increase insulin sensitivity (Hevener et al., 2007; Odegaard et al., 2008; Vats et al., 2006). However, the molecular mediators remain incompletely understood. In this study, we demonstrate that the NLRP3 inflammasome directly links defective PPAR $\gamma$  signaling to proinflammatory reprogramming in CGI-58-deficient macrophages.

Mice deficient in NLRP3 are more insulin sensitive (Stienstra et al., 2011). Increased IR in MaKO mice may be explained by CGI-58-deficiency-induced activation of the ROS-NLRP3 inflammasome pathway in macrophages. The direct consequence of NLRP3 inflammasome activation is the increased secretion of mature IL-1 family cytokines. Indirectly, these secreted proinflammatory cytokines may have driven the recruitment of other immune cells into tissues to produce more/other inflammatory cytokines. This may contribute to increases in tissue immune cells and in tissue expression or plasma concentrations of other cytokines such as iNOS, TNF- $\alpha$ , and IL-6 in MaKO mice. Increases of proinflammatory immune cells in the visceral fat were shown to positively correlate with the systemic chronic

inflammation and IR (Hotamisligil, 2006; Olefsky and Glass, 2010).

In addition to increased macrophage infiltration and polarization, the adipose tissue from the HFD-fed MaKO mice also has increased total numbers of T cells and B cells. Although the pathophysiological significance of this finding has yet to be elucidated, previous studies have shown that CD8<sup>+</sup> effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity (Nishimura et al., 2009). Recently, B cells were shown to regulate IR through modulation of T cells and proinflammatory macrophages (Winer et al., 2011). Detailed studies are needed to uncover the roles of macrophage CGI-58 in regulating T and B cell functions.

It should be emphasized that CGI-58 deficiency causes cytosolic lipid droplet accumulation, which, on one hand, may result in sequestration of proinflammatory and proapoptotic lipids or derivatives in these droplets, thereby protecting cells from lipotoxicity (Listenberger et al., 2003). On the other hand, long and oversequestration of lipotoxic lipids may eventually cause lipotoxicity. Lipotoxic lipids can activate macrophages (Priour et al., 2010) and inflammasomes (Wen et al., 2011). In the present study, although we have established a critical role of the PPAR $\gamma$ -mitochondria-ROS-NLRP3 inflammasome pathway in mediating macrophage CGI-58-deficiency-induced inflammation and IR, we have not examined the crosstalk between this pathway and the potential lipotoxic lipids in CGI-58-deficient macrophages. This certainly represents an exciting future direction.

The regulation of CGI-58 expression is largely unknown. We show in this study that LPS or saturated fatty acid downregulates CGI-58 expression in macrophages (Figure 1). This observation appears consistent with one study showing that HFD feeding reduces CGI-58 mRNA expression in the white adipose tissue of mice (Shen et al., 2007). LPS and FFAs are known to link overnutrition to obesity, IR, and type 2 diabetes (Boden et al., 1994; Kahn et al., 2006; Shi et al., 2006). It would be interesting to examine whether CGI-58 expression is suppressed in adipose tissue macrophages in humans. Additionally, increased ROS production is observed in obesity as a result of mitochondrial dysfunction (Chen and Nuñez, 2010). Future studies are needed to determine whether this is related to CGI-58 downregulation in adipose tissue macrophages.

In conclusion, macrophage CGI-58 deficiency aggravates HFD-induced inflammation and IR by activating the ROS-dependent NLRP3 inflammasome pathway through inhibition of PPAR $\gamma$ -sustained mitochondrial functions. Our findings establish macrophage CGI-58 as a suppressor of overnutrition-induced chronic inflammation and IR.

## EXPERIMENTAL PROCEDURES

### Animals and Diets

Macrophage-specific CGI-58 knockout (MaKO) mice were generated by crossing CGI-58-floxed mice (Guo et al., 2013) to the mice expressing the lysozyme promoter-driven Cre recombinase (B6.129P2-Lyz2<sup>tm1(Cre)<sup>fl</sup>/J</sup>; The Jackson Laboratory Stock #004781). All mice were housed in a specific pathogen-free environment at the University of Maryland, and all experimental procedures were approved by the Institutional Animal Care and Use Committee. Four-week-old mice were placed on either chow diet (NIH-07, Harlan Teklad Diet 7022) or a HFD (D12492, fat content 60% by calorie, Research Diets).

### Insulin Sensitivity Assessment

Glucose and insulin tolerance tests, acute tissue insulin signaling test, and hyperinsulinemic-euglycemic clamp studies were done as we have described previously (Jia et al., 2010; Shi et al., 2006).

### Methods Online

Supplemental Information include detailed methods for isolation of peritoneal macrophages (PMs) and bone-marrow-derived macrophages (BMDMs); insulin sensitivity assessment; ELISA; quantitative real-time PCR (qPCR); western blotting; isolation of splenocytes, blood cells, adipose stromal-vascular fraction (SVF) and adipose tissue macrophage (ATM); immunostaining and fluorescence-activated cell sorting (FACS); ROS assays; caspase-1 activity assays; lipid measurements; measurements of cellular ATP levels; mitochondrial biogenesis and function assays; and CGI-58 and NLRP3 gene silencing.

### Statistical Analysis

All data were expressed as mean  $\pm$  SEM and were analyzed using either one-way ANOVA or two-tailed unpaired Student's *t* test. The group difference was considered statistically significant for *p* < 0.05. For each parameter of all data presented, \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, and values not sharing a common small letter differ significantly (*p* < 0.05).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.02.047>.

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