Loss of \textit{Nlrp3} Does Not Protect Mice from Western Diet-Induced Adipose Tissue Inflammation and Glucose Intolerance

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Abstract

We tested the hypothesis that loss of \textit{Nlrp3} would protect mice from Western diet-induced adipose tissue (AT) inflammation and associated glucose intolerance and cardiovascular complications. Five-week old C57BL/6J wild-type (WT) and \textit{Nlrp3} knockout (\textit{Nlrp3}^{-/-}) mice were randomized to either a control diet (10\% kcal from fat) or Western diet (45\% kcal from fat and 1\% cholesterol) for 24 weeks (n = 8/group). Contrary to our hypothesis that obesity-mediated white AT inflammation is \textit{Nlrp3}-dependent, we found that Western diet-induced expression of AT inflammatory markers (i.e., \textit{Cd68}, \textit{Cd11c}, \textit{Emr1}, \textit{Itgam}, \textit{Lgals}, \textit{Il18}, \textit{Mcp1}, \textit{Tnf}, \textit{Ccr2}, \textit{Ccl5} mRNAs, and \textit{Mac-2} protein) were not accompanied by increased caspase-1 cleavage, a hallmark feature of NLRP3 inflammasome activation. Furthermore, \textit{Nlrp3} null mice were not protected from Western diet-induced white or brown AT inflammation. Although Western diet promoted glucose intolerance in both WT and \textit{Nlrp3}^{-/-} mice, \textit{Nlrp3}^{-/-} mice were protected from Western diet-induced aortic stiffening. Additionally, \textit{Nlrp3}^{-/-} mice exhibited smaller cardiomyocytes and reduced cardiac fibrosis, independent of diet. Collectively, these findings suggest that presence of the \textit{Nlrp3} gene is not required for Western diet-induced AT inflammation and/or glucose intolerance; yet \textit{Nlrp3} appears to play a role in potentiating arterial stiffening, cardiac hypertrophy and fibrosis.

Introduction

Current evidence implicates visceral white adipose tissue (AT) inflammation, or visceral adiposopathy [1], as a causal link between obesity and cardiometabolic complications [2–5]. However, the molecular mechanisms by which obesity promotes AT inflammation remain poorly understood. Adipocyte hypertrophy in response to caloric excess leads to infiltration of...
immune cells such as macrophages and T lymphocytes into AT, which can cross-activate one another, hence perpetuating the secretion of inflammatory cytokines from AT [4, 6–8]. AT is viewed as an active immunological organ that controls whole-body metabolism and cardiovascular function through endocrine mechanisms [2–4].

Immune cells are equipped with the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome, which is an intracellular multi-protein complex vital for the protection against numerous pathogen-derived factors [9–13]. It consists of a NOD-like receptor, the apoptosis-associated speck-like protein containing a Card (Pycard), and pro-caspase-1. The NLRP3 inflammasome can also sense metabolic "danger signals" originating from Western diet and obesity such as glucose, cholesterol, free fatty acids, uric acid, and reactive oxygen species. Activation of the NLRP3 inflammasome causes caspase-1 cleavage and subsequent secretion of pro-inflammatory cytokines, IL-1β and IL-18, and it is thought to stimulate classic pro-inflammatory (M1) polarization of macrophages [9–13]. As such, it is conceivable that obesity-induced activation of NLRP3 promotes activation and infiltration of macrophages into AT leading to increased expression of inflammatory genes. However, whether loss of Nlrp3 would protect mice from Western diet-induced AT inflammation remains largely unknown. Furthermore, given the known role of AT inflammation in instigating metabolic and cardiovascular complications [2, 3], we also hypothesized that deletion of Nlrp3 would alleviate Western diet-induced glucose intolerance, arterial stiffening, and cardiac hypertrophy and fibrosis.

Materials and Methods
Experimental Design

Male C57BL6J wild-type (WT, n = 16; Stock 000664) and Nlrp3 knockout (Nlrp3<sup>−/−</sup>, n = 16; B6.129S6-Nlrp3<sup>−/−</sup>tm1Bhk/J; Stock 021302) mice on a C57BL6J background were purchased from Jackson Laboratory (Bar Harbor, ME). WT and Nlrp3<sup>−/−</sup> mice were not littermates. At 5 weeks of age, mice were randomized to either a control diet or Western diet (n = 8/group) ad libitum for 24 weeks. Control diet (3.85 kcal/g of food) contained 10% kcal fat, 70% kcal carbohydrate, and 20% kcal protein, with 3.5% kcal sucrose (D12110704; Research Diets Inc.). Western diet (4.68 kcal/g of food) contained 44.9% kcal fat, 35.1% kcal carbohydrate, and 20% kcal protein, with 1% cholesterol and 17% kcal sucrose (D09071604; Research Diets Inc.). All mice were pair-housed and kept at 25°C with a light cycle from 0700 to 1900 and a dark cycle from 1900 to 0700 under conventional (i.e., non-specific pathogen free) animal housing facilities. At 29 weeks of age, mice were euthanized via CO₂ inhalation following a 5-hour fast. Samples were harvested and stored at -80°C until analysis. Male mice were used in this study because they are more susceptible to Western diet-induced AT inflammation [14]. All procedures were approved in advance by the University of Missouri Institutional Animal Care and Use Committee.

Total Energy Expenditure

Using a metabolic monitoring system (Promethion, Sable Systems Int., Las Vegas, NV), total energy expenditure during the 12-hour light and 12-hour dark cycles were determined by monitoring oxygen consumption and carbon dioxide production over a 3-day period, as previously described [15]. Total energy expenditure was calculated using body weight as covariate according to current recommendations [16]. These measurements were performed at 15 weeks of age.

Glucose Tolerance Testing

Glucose tolerance tests were performed at 15 and 25 weeks of age. Briefly, after a 5-hour fast, blood glucose was measured from the tail vein. The tail was nicked and blood was sampled by a
A baseline measure of blood glucose was taken prior to giving a sterile solution of 50% dextrose (2g/kg body weight (BW)) via intraperitoneal injection, as previously performed. Glucose measures were taken 15, 30, 45, 60 and 120 minutes after the glucose injection. Glucose area under curve (AUC) from baseline was calculated.

Aortic Stiffness by In Vivo Pulse Wave Velocity
Doppler ultrasound (Indus Mouse Doppler System, Webster, TX) was used as previously described [17] to evaluate pulse wave velocity (PWV), the gold standard technique for in vivo determination of arterial stiffness. Prior to sacrifice, isoflurane-anesthetized mice (1.75% in 100% oxygen stream) were placed supine on a heating board and legs secured to ECG electrodes. Determination of PWV is based on the transit time method calculated as the difference in arrival times of a Doppler pulse wave at two locations along the aorta a known distance apart. Each of the pulse wave arrival times is measured as the time from the peak of the ECG R-wave to the leading foot of the pulse wave at which time velocity begins to rise at the start of systole. The distance between the two locations along the aorta is measured with a ruler and divided by transit time. Data are expressed in cm/s. Velocity waveforms were acquired at the aortic arch followed immediately by measurement at the descending aorta proximal to the iliac bifurcation.

Fasting Blood Parameters
Blood was obtained at time of sacrifice following a 5-hour fast. Plasma glucose, cholesterol, triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and non-esterified fatty acids (NEFA) assays were performed by a commercial laboratory (Comparative Clinical Pathology Services, Columbia, MO) on an Olympus AU680 automated chemistry analyzer (Beckman-Coulter, Brea, CA) using assays according to manufacturer’s guidelines. Plasma insulin concentrations were determined using a commercially available, mouse-specific ELISA (Alpco Diagnostics, Salem, NH). Plasma concentration of IL-18 was assessed using a mouse specific ELISA (Medical and Biological Laboratories, product #7625). Plasma concentrations of IL1β, MCP-1, TNF-α, and IL-6 were assessed using a mouse-specific multiplex cytokine assay (Millipore Milliplex; Billerica, MA, USA) on a MAGPIX instrument (Luminex Technologies; Luminex Corp., Austin, TX, USA) according to the manufacturer’s instructions. Unfortunately, plasma levels of IL1β, MCP-1, and TNF-α were below the detection limit of this assay, thus only IL-6 data are presented. The whole blood samples were analyzed for HbA1c using a boronate affinity HPLC method, Trinity ultra2 (Kansas City, MO), as previously described [18].

Histological Assessments
Formalin-fixed retroperitoneal AT and liver samples were processed through paraffin embedment, sectioned at 5 μm, stained with hematoxylin and eosin (H&E) for morphometric determinations. AT samples were also stained for Mac-2 (CL8942AP, Cedarlane), a macrophage marker. Sections were evaluated via an Olympus BX34 photomicroscope (Olympus, Melville, NY) and images were taken via an Olympus SC30 Optical Microscope Accessory CMOS color camera. Adipocyte size was calculated based on 100 adipocytes/animal from three 10x fields of view, as performed previously [18]. Briefly, cross-sectional areas of the adipocytes were obtained from perimeter tracings using Image J software (NIH public domain; National Institutes of Health, Bethesda, MD). Objective quantification of macrophage infiltration was done by determining the positive Mac-2 stained area per 10x field of view using Image J.
software. The average of three 10x fields of view was used per animal. Cardiomyocyte hypertrophy was measured as previously described [19, 20]. Briefly, 5μm formalin-fixed, paraffin-embedded cardiac sections were stained with WGA conjugated to Alexa Fluor 647 (Life Technologies). Two to three images per section were captured using a Leica DMI4000B inverted confocal microscope at 40x. The smallest transverse widths (cardiomyocyte diameter) were measured of ~45–50 cross-sections of cardiomyocytes from each animal and averaged, using Image J Software. Cardiac fibrosis was determined from trichrome blue-stained slides. The positive trichrome blue-stained area per 10x field of view was manually traced using Image J software. The average of three 10x fields of view was used per animal. All histological procedures and analysis were performed by an investigator who was blinded to the groups.

Liver Triglycerides

Hepatic triacylglycerol (TG) concentration was determined using a commercially available kit (Wako L-Type TG M; Wako Pure Chemical Industries, Osaka, Japan). A BioTek uQUANT microplate spectrophotometer (Biotek Instruments, Winooski, VT) was used to analyze the absorbance set at a wavelength of 582 nm. Data are expressed as milligrams TG/gram of liver (wet weight), as described previously [18].

Gene Expression in Adipose Tissue and Stromal Vascular Cells Isolated from Adipose Tissue

Assessment of gene expression via real-time RT-PCR was performed in retroperitoneal white AT, interscapular brown AT, and the stromal vascular cell fraction freshly isolated from epididymal white AT. Stromal vascular cells were isolated via collagenase digestion as previously described [21]. All samples were homogenized and lysed in TRIzol solution using a tissue homogenizer (TissueLyser LT, Qiagen, Valencia, CA). Total RNA was isolated according to the Qiagen’s RNeasy lipid tissue protocol and assayed using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) to assess purity and concentration. The MU DNA Core confirmed our RNA extraction protocol produces optimal RNA integrity (all RQN’s > 8.0). First-strand cDNA was synthesized from total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). Quantitative real-time PCR was performed as previously described using the ABI StepOne Plus sequence detection system (Applied Biosystems). Primer sequences were designed using the NCBI Primer Design tool and have been previously published [18]. All primers were purchased from IDT (Coralville, IA). A 20 μL reaction mixture containing 10 μL iTaq Universal SYBR Green SMX (BioRad, Hercules, CA) and the appropriate concentrations of gene-specific primers plus 4 μL of cDNA template were loaded in a single well of a 96-well plate. All PCR reactions were performed in duplicate under thermal conditions as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. A dissociation melt curve analysis was performed to verify the specificity of the PCR products. Gapdh was used as house-keeping control gene. Gapdh cycle threshold (CT) was not different among the groups of animals. mRNA expression values are presented as $2^{-ΔCT}$ whereby $ΔCT = Gapdh CT - gene of interest CT$. mRNA levels were normalized to the WT control diet group.

Western Blotting

Triton X-100 tissue lysates were used to produce Western blot-ready Laemmli samples. Protein samples (10 μg/lane) were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with Mac-2 (#12733, 1:1000, Cell Signaling) and pro-caspase-1 and p10 caspase-1 (SC-514, 1:500, Santa Cruz). Intensity of individual protein bands were
quantified using FluoroChem HD2 (AlphaView, version 3.4.0.0), and expressed as ratio to control band GAPDH (#5174, 1:1000, Cell Signaling) or β-tubulin (#2146, 1:1000, Cell Signaling).

**Statistical Analysis**

A 2x2 (diet x genotype) analysis of variance (ANOVA) was used to evaluate the effects of Western diet and Nlrp3 deficiency on all dependent variables. Main effects and diet by genotype interaction were examined, and Fisher’s LSD post-hoc test was used for pair-wise comparisons. All data are presented as mean ± standard error (SE). For all statistical tests, the alpha level was set at 0.05. All statistical analyses were performed with SPSS V23.0.

**Results**

As shown in Fig 1, independent of genotype, mice fed a Western diet were heavier and had greater adiposity compared to control diet-fed mice (p<0.05). A trend was observed for Western diet-fed Nlrp3−/− mice to have greater body weights than WT counterparts, however, this difference did not reach statistical significance (p = 0.144). Western diet-fed Nlrp3−/− mice consumed more kilocalories per week than WT over the 2-week period of food intake assessment (Table 1, p<0.05). No significant effects of genotype were observed in total energy expenditure (using body weight as covariate [16]) during the light cycle (WT control diet = 3.5±0.23 vs. Nlrp3−/− control diet = 3.74±0.23 kcal/h, p = 0.475; WT Western diet = 4.23±0.07 vs. Nlrp3−/− Western diet = 4.19±0.08 kcal/h, p = 0.756) or dark cycle (WT control diet = 5.24±0.19 vs. Nlrp3−/− control diet = 4.78±0.17 kcal/h, p = 0.109; WT Western diet = 5.20±0.12 vs. Nlrp3−/− Western diet = 5.13±0.13 kcal/h, p = 0.681). Independent of genotype, compared to control-fed mice, Western diet-fed mice had greater plasma total cholesterol, low density lipoprotein, high density lipoprotein, alanine aminotransferase, aspartate aminotransferase, insulin, and HOMA-IR (Table 1, p<0.05). HbA1c was increased with Western diet in both WT and knockout mice (p<0.05); however, knockout mice exhibited slightly but significantly lower levels of HbA1c compared to WT mice, independent of diet (Table 1, p<0.05). Notably, plasma IL-18, a hallmark of NLRP3 inflammasome activation, was reduced in Nlrp3−/− mice fed control and Western diets (Table 1, p<0.05).

As illustrated in Fig 2A and 2C, adipocyte size in retroperitoneal white AT was increased with Western diet, independent of genotype (p<0.05). Similarly, levels of Mac-2 expression, examined by immunohistochemistry (Fig 2A and 2D) and Western blotting (Fig 2E), were increased with Western diet in both WT and knockout mice (p<0.05). In addition, ablation of Nlrp3 did not alter expression of other components of NLRP3 inflammasome and inflammatory cytokines in stromal vascular cells isolated from white AT of Western diet-fed mice (Fig

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**Fig 1.** Body weight and composition in WT and Nlrp3−/− mice fed a control diet versus Western diet. (A) Weekly body weights; (B) final body weight; (C) final % body fat. Data are expressed as means ± SE. WT, wild-type; KO, Nlrp3 knockout, CD, control diet; WD, Western diet; D, main effect of diet; G, main effect of genotype; DxG, diet by genotype interaction. Significant p values (<0.05) are highlighted in bold.

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Table 1. Animal characteristics.

| Variable                        | Control diet | Western diet | Control diet | Western diet | Diet effect | Genotype effect | Interaction |
|---------------------------------|--------------|--------------|--------------|--------------|-------------|----------------|-------------|
| Kcal consumed/week              | 85.5±3.5     | 90.6±2.8     | 80.5±3.6     | 99.7±3.1     | P = 0.001    | P = 0.544       | P = 0.041    |
| Retroperitoneal AT weight (g)   | 0.21±0.03    | 0.46±0.03    | 0.20±0.04    | 0.43±0.03    | P <0.001     | P = 0.503       | P = 0.768    |
| Interscapular brown AT weight (g)| 0.18±0.03    | 0.24±0.02    | 0.17±0.02    | 0.27±0.03    | P = 0.001    | P = 0.479       | P = 0.419    |
| Heart weight/body weight (%)    | 0.44±0.01    | 0.34±0.01    | 0.44±0.02    | 0.34±0.01    | P <0.001     | P = 0.962       | P = 0.782    |
| Total cholesterol, mg/dL        | 114.3±7.7    | 239.5±22.1   | 108.5±8.4    | 249.9±29.8   | P <0.001     | p = 0.910       | p = 0.687    |
| LDL cholesterol, mg/dL         | 4.7±0.4      | 21.5±3.3     | 4.3±0.3      | 27.0±5.7     | P <0.001     | p = 0.454       | P = 0.384    |
| HDL cholesterol, mg/dL         | 58.2±3.6     | 98.5±6.1     | 58.4±3.6     | 94.4±6.1     | P <0.001     | p = 0.712       | P = 0.682    |
| Triglycerides, mg/dL            | 106.7±9.2    | 112.6±38.1   | 100.5±15.8   | 89.7±13.1    | p = 0.921    | P = 0.554       | P = 0.732    |
| NEFA, mmol/L                    | 0.75±0.06    | 0.39±0.03    | 0.83±0.06    | 0.40±0.02    | P <0.001     | P = 0.343       | P = 0.527    |
| ALT, U/L                        | 165.2±81.0   | 504.9±133.9  | 78.3±16.4    | 748.3±149.1  | p <0.001     | p = 0.497       | P = 0.158    |
| AST, U/L                        | 249.4±92.6   | 319.1±61.0   | 175.5±18.0   | 397.6±71.6   | P = 0.026    | P = 0.971       | P = 0.228    |
| Insulin, ng/mL                  | 1.2±0.3      | 1.8±0.3      | 1.1±0.2      | 2.7±0.6      | P <0.004     | P = 0.269       | P = 0.224    |
| Glucose, mg/dL                  | 269.8±31.7   | 320.3±28.3   | 265.5±23.2   | 320.7±27.8   | p = 0.069    | P = 0.945       | P = 0.932    |
| HOMA-IR                         | 24.0±7.2     | 42.4±7.0     | 23.3±4.9     | 64.9±15.2    | P = 0.003    | P = 0.251       | P = 0.222    |
| Hba1c, %                        | 4.42±0.06    | 4.68±0.04    | 4.19±0.05    | 4.59±0.05    | P <0.001     | P = 0.004       | P = 0.181    |
| IL-18, pg/mL                    | 296.9±48.1   | 302.5±31.6   | 249.6±27.8   | 203.0±19.1   | p = 0.530    | P = 0.031       | P = 0.424    |
| IL-6, pg/mL                     | 148.5±87.1   | 161.5±91.7   | 51.1±24.2    | 41.1±12.3    | p = 0.982    | P = 0.103       | P = 0.860    |

Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; NEFA; non-esterified fatty acids; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HOMA-IR, homeostatic model assessment insulin resistance index. Data are expressed as means ± SE.D. Significant p values (<0.05) are highlighted in bold.

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2B). As confirmation of our model, Nlrp3 mRNA levels were undetectable in Nlrp3 null mice. Fig 3 summarizes gene expression data in retroperitoneal white AT (panel A) and interscapular brown AT (panel B). Western diet significantly increased white AT expression of the components of the NLRP3 inflammasome (Nlrp3, Pycard, Caspase-1 mRNAs), markers of macrophage infiltration (i.e., Cd68, Cd11c, Emr1, Itgam, and Lgals mRNAs) and inflammation (i.e., Il18, Mcp1, Tnfa, Ccr2, Ccls mRNAs). However, contrary to our hypothesis, these inflammatory effects were not accompanied by increased caspase-1 cleavage (Fig 2G; p >0.05). Furthermore, markers of white AT inflammation were not reduced in Nlrp3−/− relative to WT mice (Fig 3A; p >0.05). Also in both genotypes, Western diet led to increased expression of Il1β, Mcp1, Leptin, Cx11c, and Cd68 mRNAs (p <0.05) in brown AT.

Western diet increased liver weight in both WT and Nlrp3 null mice and this increase was greater in the latter (Fig 4A; interaction p <0.05). Liver triglycerides were similarly increased in both WT and knockout mice (Fig 4B; p <0.05). Fig 5 depicts results from glucose tolerance testing. Glucose tolerance was impaired with Western diet at both time points, independent of genotype; however, the impairment was more apparent at 15 weeks of age. That is, mice fed a control diet, but not Western diet, became more glucose intolerant overtime, such that differences between control and Western diet at age 25 were less pronounced.

Aortic PWV, a marker of arterial stiffness, was increased with Western diet in WT mice (Fig 6A; p <0.05 for pairwise comparison between control and Western diets in WT mice). In contrast, Nlrp3−/− mice did not exhibit an increase in aortic PWV with Western diet (p >0.05), such that the diet by genotype interaction effect was trending (p = 0.106). Western diet produced an increase in cardiomyocyte size in both WT and knockout mice (Fig 6C; p <0.05). However, the cardiomyocyte size was less in Nlrp3−/− mice compared to WT mice, independent of diet (Fig 5; p <0.05) (i.e., significant main effect of diet and genotype without diet by
Fig 2. Visceral white AT characterization in WT and Nlrp3<sup>-/-</sup> mice fed a control diet versus Western diet. (A) Representative immunohistochemical 10X images of retroperitoneal white AT stained for Mac-2; (B) gene expression in stromal vascular cells isolated from epididymal white AT; (C) average adipocyte size in retroperitoneal white AT; (D) Mac-2 positive immunostained area in retroperitoneal white AT; (E) protein content of Mac-2 via Western blotting in retroperitoneal white AT; (F) protein content of pro-caspase-1 via Western blotting in retroperitoneal white AT; (G) protein content of p10 caspase-1 (cleavage) via Western blotting in retroperitoneal white AT; (H) representative Western blot bans. Data are expressed as means ± SE. WT, wild-type; KO, Nlrp3 knockout; CD, control diet; WD, Western diet; G, main effect of genotype; DxG, diet by genotype interaction. Significant p values (<0.05) are highlighted in bold. * denotes p<0.05 in panel B.

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genotype interaction). Furthermore, cardiac fibrosis was less in \textit{Nlrp3}^{-/-} mice compared to WT mice, independent of diet (Fig 6D; \(p<0.05\)) (i.e., significant main effect of genotype without diet by genotype interaction).

### Discussion

Counter to our hypotheses, we found that Western diet-induced white AT inflammation was not accompanied by increased caspase-1 cleavage or increased circulating levels of IL-18, both characteristic features of NLRP3 inflammasome activation. Furthermore, AT inflammation and glucose intolerance caused by Western diet were not attenuated in \textit{Nlrp3}^{-/-} mice. However, \textit{Nlrp3}^{-/-} mice were protected against Western diet-induced aortic stiffening, exhibited smaller cardiomyocytes, and had reduced cardiac fibrosis compared to WT mice, under both dietary conditions. Collectively, these findings suggest that presence of the \textit{Nlrp3} gene is not required for Western diet-induced AT inflammation and glucose intolerance; yet, \textit{Nlrp3} appears to play a role in regulating arterial stiffening, cardiac hypertrophy and fibrosis. This finding is important because it identifies a role for \textit{Nlrp3} in the etiology of Western diet-induced cardiovascular impairments that is independent of AT or even systemic metabolic health.

### AT Inflammation Caused by Western Diet is Not Accompanied by Increased Caspase-1 Cleavage and it is Not Attenuated with Loss of Nlrp3

Chronic low-grade systemic inflammation, or metaflammation, underlies the pathogenesis of obesity-associated insulin resistance and cardiovascular disease [2–5, 22]. An important source

![Fig 3. AT gene expression in WT and Nlrp3^{-/-} mice fed a control diet versus Western diet.](image-url)
Fig 4. Liver characterization in WT and Nlrp3−/− mice fed a control diet versus Western diet. (A) Liver weight; (B) liver triglycerides (TG); representative histological 20X images stained for H&E. Data are expressed as means ± SE. WT, wild-type; KO, Nlrp3 knockout, CD, control diet; WD, Western diet; D, main effect of diet; G, main effect of genotype; DxG, diet by genotype interaction. Significant p values (<0.05) are highlighted in bold.

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of inflammatory cytokines is AT [2, 3]. AT expansion with caloric excess leads to infiltration of immune cells into AT, exacerbating AT inflammation and, consequently, increasing the secretion of inflammatory cytokines from AT [4, 6–8]. A better understanding of molecular mechanisms regulating AT inflammation is important as it may elucidate new therapeutic targets. Herein, we tested the hypothesis that NLRP3 inflammasome is involved in the regulation of AT inflammation. However, contrary to this notion, we found that 24 weeks of Western diet-induced expression of visceral white AT inflammatory markers was not accompanied by increased caspase-1 cleavage, a marker of NLRP3 activation. Furthermore, Nlrp3 null mice were not protected from Western diet-induced white AT inflammation relative to WT mice. To determine if the lack of effect of Nlrp3 deletion on AT inflammation was limited to white AT, we examined a subset of the same genes in interscapular brown AT. Similar to white AT, we found no evidence of reduced inflammation in brown AT from Nlrp3−/− mice. Together,
Fig 6. Aortic stiffness and cardiac characterization in WT and Nlrp3\(^{-/-}\) mice fed a control diet versus Western diet. (A) Aortic pulse wave velocity; (B) representative histological cardiac 10X images stained for Nlrp3 and Cardiometabolic Function.
these findings indicate that Nlrp3 gene is not required for Western diet-induced AT inflammation, suggesting that other mechanisms, independent of Nlrp3, mediate AT inflammation with obesity.

Glucose Intolerance Caused by Western Diet is Not Attenuated with Loss of Nlrp3

AT inflammation is an important determinant of whole body insulin resistance. Accordingly, given that lack of Nlrp3 did not abrogate Western diet-induced AT inflammation, it is not entirely surprising that glucose intolerance and increased fasting HOMA-IR, both indicators of insulin resistance, were not reduced in Nlrp3−/− mice relative to WT mice. This finding that ablation of Nlrp3 did not protect mice from obesity-induced insulin resistance is in contrast with other reports [23]. Vandanmagsar et al. [23] found that Nlrp3−/− mice fed a 60% high-fat diet (HFD) exhibited lower insulin resistance, as assessed by insulin and glucose tolerance testing, than WT mice fed the same HFD. While it is difficult to reconcile this divergence in findings, it is possible this is partly attributable to the differences in diets used to promote obesity and insulin resistance. Perhaps a critical inflammatory threshold is needed for loss of Nlrp3 to produce a phenotype in AT. In other words, the 60% HFD likely produced more inflammation and, at that critical level, the protective effect of loss of Nlrp3 could be appreciated. Alternatively, it is possible that increased whole body insulin sensitivity with elimination of Nlrp3 only manifests when mice are kept in a specific-pathogen free barrier facility. As described [24], these facilities involve ventilated cage racks that deliver HEPA filtered air to each cage with free access to sterile water through a hydropac system. Along these lines, under conventional (i.e., non-specific pathogen free) animal housing facilities, it has been shown that inflammasome deficiency-related changes in the composition of the gut microbiota (i.e., dysbiosis) leads to exacerbated hepatic steatosis as well as multiple other aspects of metabolic syndrome such as weight gain and impaired glucose homeostasis [25]. This counterintuitive increased progression of non-alcoholic steatopathy reported in Nlrp3−/− mice is even transmissible to WT mice that are co-housed with Nlrp3−/− mice [25]. While in our study gut microbiota was not examined, it remains possible that Nlrp3 deficiency-associated dysbiosis resulted in abnormal accumulation of bacterial products in the portal circulation, putting the liver at risk. In support of that hypothesis, we showed that plasma levels of ALT, a marker of liver damage, were 48% higher in Western diet fed Nlrp3−/− mice compared to WT counterparts. Further, liver weights of Western diet-fed Nlrp3−/− mice were 39% heavier than those of WT mice. Although blood and liver levels of endotoxin were not measured in this study, the increase in liver mass did not appear to be explained by increased lipid deposition as liver TGs were not different between Nlrp3−/− and WT mice (Fig 4C). This disconnect between liver weight and lipid accumulation is intriguing and requires further investigation. However, given prior evidence that Nlrp3−/− deficient mice show accelerated clearance of triglycerides after a fat challenge [26], we speculate that the disconnect between liver weight and triglycerides may be related to an accelerated clearance of triglycerides in Nlrp3−/− mice.

As expected, Nlrp3−/− mice demonstrated lower circulating levels of IL-18 than WT mice. The extent of reduction was comparable to that of some studies [27] but considerably less to that of others [23] that reported the blood IL-18 levels of Nlrp3−/− mice to be below the detection
limit of the assay. We do not have a good explanation for these discrepancies among studies; however, it is possible that differences in assay methods play a role. Regardless, it is suggestive that circulating levels of IL-18 are not exclusively regulated by Nlrp3 gene. Given that IL-18 null mice exhibit hyperphagia, obesity and insulin resistance [28], the reduced levels of IL-18 may explain the observation that Nlrp3\(^{-/-}\) mice fed a Western diet appeared to be more hyperphagic and tended to be heavier than WT mice. In this regard, there is evidence that expression of leptin receptor parallels the expression of Nlrp3 [29]. Additional studies are needed to determine if leptin signaling in the brain is reduced with loss of Nlrp3 and whether this function of Nlrp3 is inflammasome-independent [26, 30]. Taken together, it is possible that our finding that ablation of Nlrp3 did not protect mice from glucose intolerance may be attributable to counteracting effects from the gut in a setting of standard co-housing facilities and/or reduced IL-18 signaling. Despite this, we did find a small, but statistically significant, reduction in levels of HbA1c in Nlrp3\(^{-/-}\) mice compared to WT, independent of diet. We also noted that Nlrp3\(^{-/-}\) mice fed a Western diet had slightly greater fasting insulin levels compared to WT mice \((p = 0.19)\), suggesting a potential increase in \(\beta\)-cell function necessary for maintenance of glucose homeostasis.

**Western Diet-Induced Aortic Stiffening is Abrogated with Loss of Nlrp3**

Although Nlrp3 deficiency did not affect AT inflammation and/or glucose intolerance, we did observe that Nlrp3\(^{-/-}\) mice were protected from a Western diet-induced increase in aortic stiffness. This finding is relevant in light of evidence from humans indicating that aortic stiffness is an independent predictor of cardiovascular disease and all-cause mortality [31–33]. The protective effects of Nlrp3 deficiency on the vasculature has also been suggested by others. Duewell et al. [27] found that LDL-R deficient mice whose bone marrow-derived cells lacked Nlrp3 were resistant to the development of HFD-induced atherosclerosis. In addition, Bando et al. [34] recently showed in a cohort of 72 patients that expression of Nlrp3 in subcutaneous white AT was an independent predictor for the severity of coronary atherosclerosis. Of interest, the authors noted that AT expression of Nlrp3 was also associated with serum levels of uric acid [34], a likely contributor to arterial stiffness [35]. Given the growing evidence that aortic stiffness is an important determinant of cardiovascular disease and mortality [31–33], elucidating molecular mechanisms and targets for arterial de-stiffening is of paramount importance. More research is needed to examine vascular effects of Nlrp3 signaling in the setting of obesity and metabolic disease.

**Loss of Nlrp3 Reduces Cardiac Hypertrophy and Fibrosis**

Another interesting finding of the present study was the observation that Nlrp3 null mice exhibited smaller cardiomyocytes, unlike adipocytes, and reduced cardiac fibrosis in control and Western diets. This finding is relevant in that cardiac hypertrophy and fibrosis contribute to the progression of heart failure. In this regard, Bracey et al. [36] recently showed in mice that cardiac-specific overexpression of the calcineurin transgene (CNTg), a model of heart failure, resulted in increased cardiac expression of Nlrp3, hypertrophy and reduced fractional shortening, indicative of cardiac dysfunction. Importantly, CNTg mice bred into the Nlrp3\(^{-/-}\) background exhibited restored cardiac function [36]. There is also evidence in a rat model of type 2 diabetes that Nlrp3 gene silencing ameliorates cardiac inflammation, fibrosis, and function and that NLRP3 inflammasome activation is mediated by increased oxidative stress-induced NF-kB activation [37]. Also, it has been reported that Nlrp3-deficient mice are protected against angiotensin II-induced cardiac fibrosis [38] and that pharmacological inhibition of NLRP3 markedly reduces cardiac fibrosis and preserves systolic function after ischemic and
nonischemic injuries in mice [39]. The role of Nlrp3 in regulating fibrosis has also been reported in non-cardiac tissues including the kidney. For example, it has been shown that deletion of Nlrp3 protects renal fibrosis via inhibition of mitochondrial dysfunction [40]. These findings, combined with our data, support the role of Nlrp3 in modulating cardiac structure and function and highlight the importance of Nlrp3 as a potential therapeutic target. Although more studies are necessary to elucidate the underlying mechanisms by which Nlrp3 regulates collagen synthesis, fibrosis, hypertrophy, and cardiac function, we speculate that mitochondrial dysfunction and oxidative stress may be implicated in these processes. Specifically, it is possible that loss of Nlrp3 mitigates mitochondrial-derived production of reactive oxygen species. Because reactive oxygen species are a signal for collagen synthesis, fibrosis, and cardiovascular stiffness [41–43], the cardiovascular protection in Nlrp3 null mice may be in part attributed to reduced oxidative stress.

Considerations

Several considerations for the overall interpretation of the current findings are warranted. First, in our study, we found that 24 weeks of Western diet feeding did not significantly increase caspase-1 cleavage in AT or circulating levels of IL-18, two markers of NLRP3 inflammasome activation. Given that activated caspase-1 is secreted from cells as a function of time, it is possible that we missed the window of time during which p10 caspase-1 was increased in AT. That is, at this time, we cannot rule out the possibility that after 24 weeks of Western diet feeding caspase-1 processing and secretion may be increased. Therefore, the need for additional time-course studies evaluating the kinetics of caspase-1 cleavage and secretion from AT during Western diet would be of interest. Second, AT inflammation was assessed via RT-PCR (in whole tissue and stromal vascular cells), immunohistochemically with Mac-2 staining, and by Western blotting (Mac-2 protein content) in whole tissue. Future research using FACS analysis is needed to examine the differential inflammatory effects of obesity and Nlrp3 ablation in AT immune cells (i.e., macrophages, T-cells) vs. adipocytes. Third, while it has been described that Nlrp3 deficiency-associated dysbiosis leads to liver complications and impaired glucose homeostasis [25], our study did not characterize the microbiota or endotoxin levels, which can be considered a limitation. The mechanisms by which Nlrp3 appear to play a role in cardiovascular tissue, and its implications, remain largely elusive. Accordingly, additional studies should further characterize the cardiovascular phenotype of Nlrp3 null mice at the molecular and functional levels including measures of blood pressure, endothelial function, and cardiac function. It should also be noted that this study was conducted in male mice undergoing a 24-week control vs. Western diet intervention, kept in a non-specific pathogen free facility, and fasted for 5 hours prior to tissue harvest. In short, future studies need to establish the extent to which differences in sex, duration and composition of diets, facility conditions, and hours of fasting have an impact on these results.

Conclusion

In conclusion, findings from the present study indicate that Western diet-induced glucose intolerance and AT inflammation is Nlrp3-independent. Nonetheless, we showed that Nlrp3 is involved in the regulation of arterial stiffening and cardiac remodeling, suggesting that Nlrp3 may be a therapeutic target for the prevention and treatment of cardiovascular disease. More research is needed to understand the physiological role of Nlrp3 across organs, as very recently put forth in a review [44].

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