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Caspase-12, but Not Caspase-11, Inhibits Obesity and Insulin Resistance

Alexander M. Skeldon,* Alexandre Morizot,† Todd Douglas,§ Nicola Santoro,§ Romy Kursawe,§ Julia Kozlitina,§ Sonia Caprio,§ Wajahat Z. Mehal,‖ and Maya Saleh*‡†‡

Inflammation is well established to significantly impact metabolic diseases. The inflammatory protease caspase-1 has been implicated in metabolic dysfunction; however, a potential role for the related inflammatory caspases is currently unknown. In this study, we investigated a role for caspase-11 and caspase-12 in obesity and insulin resistance. Loss of caspase-12 in two independently generated mouse strains predisposed mice to develop obesity, metabolic inflammation, and insulin resistance, whereas loss of caspase-11 had no effect. The use of bone marrow chimeras determined that deletion of caspase-12 in the radio-resistant compartment was responsible for this metabolic phenotype. The Nlrp3 inflammasome pathway mediated the metabolic syndrome of caspase-12–deficient mice as ablation of Nlrp3 reversed Casp12−/− mice obesity phenotype. Although the majority of people lack a functional caspase-12 because of a T125C single nucleotide polymorphism that introduces a premature stop codon, a fraction of African descendants express full-length caspase-12. Expression of caspase-12 was linked to decreased systemic and adipose tissue inflammation in a cohort of African American obese children. However, analysis of the Dallas Heart Study African American cohort indicated that the coding T125C single nucleotide polymorphism was not associated with metabolic parameters in humans, suggesting that host-specific differences mediate the expressivity of metabolic disease. The Journal of Immunology, 2016, 196: 437–447.

Metabolic diseases such as type 2 diabetes and the metabolic syndrome have quickly become a major global health concern. The increasing prevalence of obesity has led to the development of these diseases in adults as well as children. Although lifestyle is a large contributor to this problem, the underlying genetic factors are less well understood, and their elucidation may thus aid in the identification of therapeutic targets. A rapidly expanding number of studies have implicated the immune system in playing a pivotal role in the development of metabolic diseases.

Obesity results from an energy imbalance, caused by excess caloric intake that exceeds metabolic requirements. This energy surplus is stored as lipids, leading to overexpansion of the adipose tissue, and consequently local inflammation, possibly initiated by hypoxia, ER stress, and/or pattern recognition receptor (PRR) detection of endogenous metabolic danger signals (1). The onset of obesity correlates with chronic inflammation of metabolic tissues, characterized by high proinflammatory cytokine secretion and elevated levels of infiltrating immune cells including macrophages, neutrophils, T cells, and B cells (1). Macrophages in particular may constitute up to 40% of the cells found in the adipose tissue of obese patients (2). Genetic studies or Ab depletion of immune cells or chemokines have implicated numerous inflammatory mediators in metabolic disease. Inflammation can lead to insulin resistance by acting directly on insulin-responsive cells, partially through the action of the inflammatory kinases INK and IKK that phosphorylate and inhibit insulin receptor substrate-1 (3, 4).

The inflammatory caspases are a family of cysteine proteases, composed of caspase-1, -4, -5, and -12 in humans and caspase-1, -11, and -12 in mice. Caspase-1, along with a PRR and the adaptor molecule ASC, is capable of forming a cytosolic multiprotein complex termed the inflammasome. Detection of exogenous or endogenous danger signals by the PRR promotes the activation of caspase-1, which triggers an inflammatory response that is primarily characterized by IL-1β and IL-18 secretion (5). Mounting evidence suggests a critical role for the Nlrp3 inflammasome as a major regulator of inflammation in metabolic diseases. In response to several metabolic danger signals, including saturated fatty

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Abbreviations used in this article: ALT, alanine transaminase; BMI, body mass index; CRP, C-reactive protein; DHS, Dallas Heart Study; DIO, diet-induced obesity; DKO, double-knockout; eFAT, epididymal adipose tissue; ES, embryonic stem; GTT, glucose tolerance test; HFD, high-fat diet; HOMA-IR, homeostatic model assessment; LFD, low-fat diet; PRR, pattern recognition receptor; qPCR, quantitative PCR; Ripk2, receptor-interacting protein kinase 2; SNP, single nucleotide polymorphism; TG, triglyceride; WAT, white adipose tissue; WT, wild-type.
acids, ceramide, islet amyloid polypeptide, and hyperglycemia (6–8). Nlrp3 assembles an inflammasome, which activates the proinflammatory protease caspase-1. Implementation of the diet-induced obesity (DIO) experimental model to mice deficient in various inflammasome components, such as Nlrp3−/−, Asc−/−, or Ice−/− mice [deficient in caspase-1 and carrying a null mutation in caspase-11 (9, 10)], has demonstrated that inflammasome signaling alters mouse susceptibility to high-fat diet (HFD)-induced insulin resistance. Whereas many studies have demonstrated that caspase-1−/−dependent IL-1β production is the triggering pathological mechanism in insulin resistance, it is debated whether inflammasome signaling regulates obesity per se and how. For instance, although some reported that the weight of Nlrp3−/− mice was comparable with that of wild-type (WT) controls in the DIO model (7, 8), others described Nlrp3−/−, Asc−/−, and Ice−/− mice leaner in phenotype (11, 12), possibly mediated by decreased intestinal lipid absorption (13) or increased lipid oxidation (11). Furthermore, it was shown that the inflammasome pathway might affect obesity indirectly through effects on the host microbiota (14). Although the inflammasome is primarily studied in cells of hematopoietic origin, it may play a role in both immune cells and stromal cells in the context of metabolic disease. The radiosensitive compartment was determined to be important for the insulin sensitivity observed in Asc−/− mice (8). However, bone marrow chimera experiments have also indicated that caspase-1 is active in radio-resistant cells, where it mediates host lipid metabolism (15). The precise roles of the inflammasome and its regulation of obesity and metabolic disease have not yet been fully determined.

Unlike Caspase-1, little is known about how the remaining inflammatory caspases affect metabolic disease. Caspase-11 has recently been established to recognize intracellular LPS and Gram-negative bacterial pathogens, leading to activation of a noncanonical inflammasome (9). A role of caspase-12, as an immunomodulatory factor, has been primarily studied in the context of infections and exposure to microbial ligands (16–19); however, little is known regarding its function in sterile inflammation, such as that elicited in obesity. Whereas Caspase-1 can cleave a variety of substrates (20), the only known substrate of rodent Caspase-12 is itself (21), although this proteolytic activity was demonstrated to be unnecessary for its regulation of innate immune pathways (18). Human Caspase-12 likely lacks this enzymatic activity, because of an SHG (Ser-His-Gly motif) to SHS (Ser-His-Ser) mutation in the catalytic domain (22). Interestingly, a premature stop codon in human Caspase-12 prevents its expression in the majority of the human population or its continued maintenance.

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Given the importance of the contribution of innate immunity to metabolic diseases (1), we investigated the role of caspase-11 and caspase-12 in obesity and insulin resistance in mice and humans. In this study, we demonstrate that mice ablated for caspase-12 develop spontaneous obesity and insulin resistance. This is dependent on the Nlrp3 inflammasome, although interestingly independent of the radio-sensitive compartment. Analysis of the effect of the CASP12 T125C SNP in the Dallas Heart Study (DHS) African American cohort (25), however, suggested that a functional caspase-12 allele does not correlate with improved metabolic parameters in humans, although in a small cohort of African American obese children, expression of caspase-12 was associated with dampened inflammatory markers.

Materials and Methods

Animal experiments

Mice were housed at room temperature with a 12-h light/dark cycle with food and water provided ad libitum. The mice were fed either a standard chow diet (low-fat diet [LFD]) (2020x Teklad Rodent Diet; 16% calories from fat; 3.1 kcal/g) or a HFD (Research Diet D12451; 45% kcal from fat; 4.73 kcal/g). HFD feeding was initiated in mice at 6 wk of age.

Mouse strains

Casp11−/−, Casp12−/−/Asc−/−, Ice−/−, Nlrp3−/−, and Ripk2−/− mice have been previously described (9, 10, 18, 26, 27). Casp12−/−/Asc−/− mice are from Deltagen but are not direct descendants of the Merck Casp12−/−/Asc−/− mice used in Saleh et al. (18). Casp12−/−/Ripk2−/− and Casp12−/−/Nlrp3−/− were generated for this study. Casp12−/−/Ripk2−/− (Casp12−/−/Ripk2−/−/Asc−/−) mice were generated on a B6 background by the Wellcome Trust Sanger Institute. All experiments were performed under guidelines of the animal ethics committee of McGill University (Montreal, QC, Canada).

Glucose tolerance test and insulin tolerance test

Age-matched male mice were fasted for 6 h before i.p. injection with 2 g/kg dextrose (LFD and HFD) or human recombinant insulin (Humulin, Eli Lilly) 0.75 mU/g (LFD) or 2.0 mU/g (HFD). Blood glucose levels were measured from the tail vein using a OneTouch Ultra 2 glucometer.

Western blots

Tissues were lysed in buffer B150 (20 mM Tris-HCl pH 8.0, 150 mM KCl, 10% glycerol, 5 mM MgCl2, and 0.1% Nonidet P-40) supplemented with Complete mini protease inhibitors (catalog no. 11836153001; Roche Applied Science) and phosphatase inhibitors (catalog no. S7920, 71768, G6376; Sigma). Protein lysates were separated on SDS-PAGE and transferred to nitrocellulose membrane. Blots were probed with Abs against caspase-1 and -12, Casp11, and Nlrp3 as described in Saleh et al. (18).

ELISAs and serum analysis

Cytokines were determined using the following ELISA kits: IL-6 (catalog no. DY406; R&D); IL-18 (catalog no. 1625; MBL International), KC (catalog no. DY453; R&D), and MCP-1 (catalog no. DY479; R&D). Serum alanine transaminase (ALT) and cholesterol were determined by a Vitros 250/350 machine.

Dual-energy x-ray absorptiometry

Fat and lean mass were calculated using a GE Lunar PIXImus machine.

Hepatic triglyceride analysis

Hepatic lipids were extracted using a modified Bligh-Dyer extraction protocol. Approximately 200 mg liver tissue was homogenized in a 1:2.5/1.25 (v/v) mixture of 0.5M acetic acid/methanol/chloroform. The mixture was shaken and 1.25 vol chloroform was added. After overnight shaking, 1.25 vol 0.5M acetic acid was added and the samples were spun at 1500 × g. The organic phase was collected, dried, and resuspended in isopropanol. Triglycerides (TGs) were determined using a Serum Triglyceride Determination kit (Sigma TR0100) and normalized to liver weight.

Bone marrow chimera

Age-matched male mice were irradiated and reconstituted with bone marrow cells from donor mice. Genotype was verified by PCR analysis of blood. Mice were placed on antibiotics (trimethoprim 0.2 g/l, sulfame-thoxazole 1 g/l), beginning 3 d before lethal irradiation, for 3 wk. Mice were placed on antibiotics (trimethoprim 0.2 g/l, sulfamethoxazole 1 g/l), beginning 3 d before lethal irradiation, for 3 wk. Mice were placed on antibiotics (trimethoprim 0.2 g/l, sulfamethoxazole 1 g/l), beginning 3 d before lethal irradiation, for 3 wk. Mice were placed on antibiotics (trimethoprim 0.2 g/l, sulfamethoxazole 1 g/l), beginning 3 d before lethal irradiation, for 3 wk.

Flow cytometry

Epidermal adipose tissue was excised from 26-wk-old HFD mice, minced, and incubated in 1 mg/ml Type 2 Collagenase (Sigma) for 1 h at 37 C. After RBC lysis, the stromal vascular fraction cells were counted and stained. Data were acquired on a Canto instrument (BD Biosciences) equipped for the detection of eight fluorescent parameters. The following Abs were used...
for flow cytometry analysis: anti–CD3-PerCP/Cy5.5 (145-2C11), anti–CD11b eFlour450 (M170), anti–B220-allophycocyanin (RA3-6B2), anti–CD5-PECy7 (30-F11) (all from eBioscience); anti–CD19-PECy7 (ID3), anti–GR1-allophycocyanin-Cy7 (RB6-8C5), (all from BD Biosciences); and anti–F4/80-PerCP/Cy5.5 (BM8.1) (Tonbo Biosciences).

Indirect calorimetry

Mice were placed in Oxymax-CLAMS system (Columbus Instruments) metabolic cages housed with a 12-h light/dark cycle and free access to water and food. Animals were allowed to acclimatize for 48 h before readings were taken for a 24-h period.

Immunohistochemistry

Subcutaneous biopsies from 15 subjects were used for immunohistochemical staining and CD68 was used as a marker for macrophages. Staining was performed using a standard protocol on sections from formalin-fixed, paraffin-embedded tissue blocks. Serial sections were deparaffinized, rehydrated, and treated with 10 mM citrate buffer (pH 6.0) in a steamer and then endogenous peroxidase was blocked with 3% H2O2. The sections were then incubated for 1 h at room temperature with primary Abs, mouse monoclonal anti–CD68 (Ab-3 clone KP1; Thermo Fisher Scientific, Fremont, CA). After rinsing in TBS buffer containing 0.25% Triton X-100 (pH 7.2), sections were incubated with ENVISION+ (K4007 or K4011; DAKO, Carpinteria, CA) followed by visualization with 3,3′-diaminobenzidine tetrachloride (Dako). All sections were counterstained with GILL III Hematoxylin, dehydrated, and coverslipped with a resinous mounting media. For each subject, the number of macrophages (identified as CD68+ cells) within 10 regions of interest were counted by two independent observers using a light microscope and normalized to the number of counted adipocytes.

Quantitative real-time PCR of human samples

Total RNA was isolated using TRIzol reagent and was further purified using a RNeasy kit (Qiagen, Valencia, CA). The quantification of IL-6 and TNF-α by real-time RT-PCR was performed using an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The nucleotide sequences of the primers and PCR conditions can be provided upon request. For each run, samples were run in duplicates for both the gene of interest and 18S. Quantitative analysis was determined by ΔΔCT method normalized to both a control and 18S message.

Yale pediatric cohort

The Yale Pediatric Obesity cohort is a multicentric cohort of obese children and adolescents carefully phenotyped in regard to glucose and lipids metabolism, liver function, and fat partitioning. Presently, the cohort consists of 1109 obese children and adolescents from New Haven, Connecticut, area recruited through the Yale Pediatric Obesity Clinic. For the purpose of this study, we genotyped 256 obese African American children and adolescents with a mean age of 14.1 ± 3.7 and a mean z-score body mass index (BMI) of 2.33 ± 0.52 who underwent an oral GTT and the measure of plasma C-reactive protein (CRP) and IL-6 as described earlier (28). A subgroup of 15 of them (mean age, 14.8 ± 3.4 years; mean z-score BMI, 2.0 ± 0.4) underwent a s.c. fat biopsy (29). All the patients were genotyped for the T125C variant by automatic sequencing using the following primers: 5′-ATATAATTCCTATAATATCATAC-3′ and 5′-GTC-TAAACTCTCCACACACCT-3′ (TA 55°C).

DHS analysis

DHS is a longitudinal, multiethnic, population-based probability sample of Duke County, North Carolina African Americans were oversampled to comprise ~50% of the population. Details of the study design and recruitment procedures have been previously described (25). The study was approved by the institutional review board of the University of Texas Southwestern Medical Center, and all participants provided written, informed consent. The present investigation includes all African American participants of the DHS who provided fasting blood samples during the initial enrollment (2000–2004) and a subgroup of 15 of them (mean age, 14.8 ± 3.4 years; mean z-score BMI, 2.0 ± 0.4) underwent a s.c. fat biopsy (29). During each examination, participants completed a detailed staff-administered survey, which included questions about demographics, socioeconomic status, medical history, and current medication use, and underwent a health evaluation that involved measurement of blood pressure, anthropometry, blood and urine sample collection, and imaging studies. BMI was calculated as weight in kilograms divided by height in meters squared. Blood lipid and lipoprotein levels were measured using standard enzymatic methods. Insulin resistance was quantified from fasting blood glucose and insulin levels using homeostatic model assessment (HOMA-IR). Diabetes was defined as a self-reported physician diagnosis of diabetes, use of glucose-lowering medication, or fasting glucose ≥126 mg/dl. Hepatic TG content was measured using hydrogen proton-magnetic resonance spectroscopy in a subset of 1106 African Americans participants, who completed the initial clinic visit (30). Genotyping was performed using Illumina Infinium Human Exome-12 v1_A BeadChip. Genotype calling and quality control have been previously described (31). CASP12 genotypes were in Hardy–Weinberg equilibrium (p = 0.80). The association between CASP12 T125C genotype and clinical phenotypes was tested using linear regression adjusted for age, sex, BMI, and type 2 diabetes mellitus, where necessary. We applied a logarithm transformation to BMI, HOMA-IR, and TGs, and a power transformation to hepatic TG content before analysis to achieve approximate normality of the residuals. Diabetic individuals were excluded from selected analyses as indicated.

Results

Caspase-12−/−mice develop obesity and insulin resistance on a HFD

Following the extensive evidence linking innate immunity and the inflammasome–caspase-1 pathway to metabolic disease, we sought to investigate a possible role of the related inflammatory caspases-12 and -11 in metabolic regulation. We examined two independent Casp12−/− mutant mouse lines, the first generated with 129 embryonic stem (ES) cells and then backcrossed to a B6 background (referred to as Casp12−/−(129)), and the second generated using B6 ES cells (referred to as Casp12−/−(B6)). Kayagaki et al. (9) recently identified that the 129 strain of mice harbored a null mutation in the Casp11 gene, raising the possibility that knockout mice of genes neighboring Casp11 generated with 129 ES cells might also be deficient in caspase-11. Indeed, Casp11−/− mice (herein referred to as Ice−− mice) also carry the 129S-derived Casp11 null mutation (9). Genotyping for the passenger mutation in Casp11 indicated that the Casp12−/−(129) mice in our facility are also deficient in caspase-11, whereas caspase-11 is expressed in Casp12−/−(B6) mice (Supplemental Fig. 1A). Interestingly, when placed on a HFD (45% kcal fat), both Casp12−/−(129) and Casp12−/−(B6) strains became obese compared with WT mice (Fig. 1A). Casp11−/− and Ice−− mice were similar to controls, suggesting that loss of caspase-11 had little effect on the weight gain of the Casp12−/−(129) mice. After 16 wk of HFD, caspase-12−/− deficient strains had increased inguinal, mesenteric, and perirenal adipose depot weight compared with WT mice (Fig. 1B). Ice−− mice had increased epididymal adipose tissue (eFat) fat pad weight in relation to all other strains; however, in our studies, eFat weight from DIO mice did not directly correlate with total body weight or the weight of other fat pads (data not shown). Casp11−/− mice were similar to controls. Liver weight was increased in Casp12−/−(129) mice, which correlated with their overall heavier body weight, while reduced in Ice−− mice compared with WT control subjects. Histological analysis of the eFAT revealed no differences in adipocyte size before analysis to achieve approximate normality of the residuals. Diabetic individuals were excluded from selected analyses as indicated.
caspase-12 also led to obesity on LFD; however, this response was confined to male but not female mice (Fig. 2A, 2B, Supplemental Fig. 1B, 1C). The difference in body-weight gain between LFD-fed Casp12<sup>−/−</sup>(129) mice and WT controls was evident starting at 12 wk of age (Fig. 2A, B). The increased weight gain in Casp12<sup>−/−</sup>(129) mice corresponded to increased adiposity, as determined by dual-energy x-ray absorptiometry scan (Supplemental Fig. 1D), increased adipose depot weight of the inguinal, eFAT, and mesenteric fat pads (Fig. 2C), and increased eFAT adipocyte hypertrophy in LFD fed mice (Fig. 2D, 2F, 2G). Although differences in liver weight and liver injury, as determined by serum ALT levels, were apparent only on HFD (Fig. 2C, 2H), both diets resulted in increased hepatic lipid deposition and TGs in Casp12<sup>−/−</sup>(129) mice compared with WT controls (Fig. 2E, 2I). In addition, serum cholesterol was more elevated in Casp12<sup>−/−</sup>(129) mice when fed an HFD (Figs. 1D, 2J). To determine whether there were metabolic abnormalities associated with caspase-12 ablation, we first determined food intake in LFD-fed mice and found that it was similar between genotypes (Supplemental Fig. 2A). Next, we performed indirect calorimetry experiments on 8-wk-old WT and Casp12<sup>−/−</sup>(129) mice fed an LFD. We selected this time point because it is before the onset of differential body weight gains in the two genotypes to assess causative rather than consequential effects of obesity (Supplemental Fig. 2B). We observed no significant differences in respiration (VO<sub>2</sub>, CO<sub>2</sub>), energy expenditure, or movement in the cages (Supplemental Fig. 2C–H). However, after HFD, Casp12<sup>−/−</sup>(129) mice had reduced respiration and movement (Supplemental Fig. 2I–O), suggesting that after the onset of obesity, metabolic abnormalities may begin to contribute to disease. Altogether, these results indicate that loss of caspase-12 in mice results in spontaneous obesity that is exacerbated with HFD feeding leading to fatty liver disease. This phenotype, however, is not a result of drastic intrinsic metabolic abnormalities in Casp12<sup>−/−</sup>(129) mice.

Caspase-12-deficient mice develop glucose intolerance and insulin resistance

There is a significant correlation between obesity and metabolic diseases in humans and rodent models, particularly between obesity-induced metabolic inflammation and insulin resistance. To determine whether obese Casp12<sup>−/−</sup>(129) mice became insulin resistant, we performed glucose tolerance tests (GTTs) and insulin tolerance tests. This analysis demonstrated that at ∼20 wk of age, Casp12<sup>−/−</sup>(129) mice developed glucose intolerance and insulin resistance both when fed an LFD (Fig. 3A, 3B) and an HFD (Fig. 3C, 3D). HFD-fed Casp12<sup>−/−</sup>(129) mice also had impaired
tolerance to glucose compared with WT controls (Fig. 3E), whereas loss of caspase-11 or caspase-1 did not have an effect at this time point (Fig. 3F). Analysis of the epididymal fat revealed reduced expression of the insulin-sensitive genes Adiponectin and Ppar-α in caspase-12–deficient mouse strains, but not in Casp11<sup>−/−</sup> mice (Fig. 3G). Binding of insulin to its receptor leads to a downstream signaling cascade, resulting in phosphorylation of AKT. To assess insulin signaling in metabolic tissues, we injected HFD-fed mice i.p. with a 5.0 U/kg bolus of insulin, and levels of phosphorylated AKT in white adipose tissue (WAT), muscle, and liver were analyzed 10 min later by immunoblot analysis. Fig. 3H shows that Casp12<sup>−/−</sup> mice exhibited reduced insulin signaling in all three metabolic tissues.

Ablation of Caspase-12 in the radio-resistant compartment leads to obesity

To further delineate how caspase-12 may be contributing to the development of obesity, we generated bone marrow chimeras. Interestingly, HFD-fed Casp12<sup>−/−</sup> recipient mice developed increased body weight, adiposity, and liver weight irrespective of whether they were transplanted with bone marrow from WT or Casp12<sup>−/−</sup> donors (Fig. 4A, 4B). Thus, the genotype of the transplanted hematopoietic cells had little bearing on the obesity phenotype of Casp12<sup>−/−</sup> mice. In agreement with this finding, Caspase-12 could not be detected in the radio-sensitive compartment of adipose tissue (Supplemental Fig. 3A). Similarly, loss of caspase-12 in the radio-resistant compartment was responsible for mediating the glucose intolerance phenotype (Fig. 4C). These results suggested that both obesity and insulin resistance in Casp12<sup>−/−</sup> mice are likely independent of bone marrow–derived immune cells but instead may be linked to the function of caspase-12 in radioresistant cells.

Caspase-12 deficiency leads to increased WAT inflammation

Given our previous findings that caspase-12 can regulate various inflammatory pathways, including caspase-1, NOD, and NF-κB signaling (16–18), we examined adipose tissue inflammation in DIO WT and Casp12<sup>−/−</sup> mice. Overnight organ culture of epididymal adipose tissue from Casp12<sup>−/−</sup> mice after HFD revealed enhanced secretion of IL-6 and, to a lesser extent, KC (Fig. 4D). Similarly, Casp12<sup>−/−</sup> mice had significantly more serum IL-18 cytokine, but not MCP-1, compared with WT mice (Fig. 4E). Consistent with increased IL-18 levels, Casp12<sup>−/−</sup> and Casp12<sup>−/−</sup> mice also exhibited increased activation of

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**FIGURE 2.** Casp12<sup>−/−</sup> mice develop spontaneous obesity on an LFD. (A) Photos of male C57BL/6 WT mice and Casp12<sup>−/−</sup> at 30 wk of age fed an LFD or HFD. (B) Body-weight curve of male mice. HFD started at 6 wk of age (n = 24–39 mice/group). (C) Relative weight of adipose depots and liver at 26 wk of age (n = 15 mice/group). (D) H&E staining of epididymal adipose tissue at 26 wk of age. Scale bar, 200 μM. (F and G) Average epididymal adipocyte area from LFD-fed mice. (H) Serum ALT (LFD WT, n = 13; LFD Casp12<sup>−/−</sup>, n = 12; HFD WT, n = 12; HFD Casp12<sup>−/−</sup>, n = 10). (I) Hepatic TGs (LFD WT, n = 14; LFD Casp12<sup>−/−</sup>, n = 13; HFD WT, n = 6; HFD Casp12<sup>−/−</sup>, n = 6). (J) Serum cholesterol. Data are presented as mean ± SEM. Statistical analysis was performed using Student’s t test. Statistical significance is presented as follows: *p < 0.05, **p < 0.01, ***p < 0.001.
caspase-1 in the epididymal adipose tissue, evident by elevated levels of the active p20 fragment, as detected by Western blot (Fig. 4F, Supplemental Fig. 3B). The epididymal adipose tissue of Casp12−/− and Casp12−/−(B6) had increased expression of CD45 suggestive of increased immune cell infiltration (Fig. 4G). We next determined levels of immune cell infiltrates in the epididymal adipose tissue stromal vascular fraction from DIO mice by flow cytometry. Quantification of the numbers of neutrophils, myeloid cells, NK cells, and T and B lymphocytes revealed a general trend of increased immune cell infiltration in the WAT of Casp12−/−(129) mice compared with WT controls, with macrophages being most significantly increased in the absence of caspase-12 (Fig. 4H, Supplemental Fig. 3C).

Ablation of Nlrp3, but not Ripk2, reverses the obesity and insulin resistance phenotype of Casp12−/−(129) mice

To define the mechanism of caspase-12 action in metabolism and metabolic inflammation and determine whether the inflammasome or Nod1/2 pathways (17, 18) were involved, we bred Casp12−/−(129) mice with Nlrp3−/− mice or mice deficient in the Nod1/2 pathways (17, 18) were involved, we bred Casp12−/−(129) mice with Nlrp3−/− mice or mice deficient in the Nod1/2 path-
with Casp12<sup>−/−</sup> mice (Fig. 5E). Casp12<sup>−/−</sup> Nlrp3<sup>−/−</sup> DKO mice were also analyzed on a HFD. These mice were leaner and more glucose tolerant compared with Casp12<sup>−/−</sup> mice (Fig. 5F, 5G). Although the Nlrp3 inflammasome is commonly studied in macrophages, there are little data regarding its role in adipocytes. Caspase-1 is expressed during adipogenesis (11) and we were able to detect inducible Nlrp3 expression in primary differentiated adipocytes (Supplemental Fig. 3D). Thus, adipocytes represent a potential radio-resistant cell type that expresses both Caspase-12 (Supplemental Fig. 1A) and Nlrp3.

The human CASP12 T<sup>125</sup> allele is associated with elevated metabolic inflammation but not with metabolic disease

An SNP in exon 4 of the human CASP12 gene at amino acid position 125 (T<sup>125</sup>) introduces a premature stop codon and precludes expression of caspase-12 from the majority of the human population. In contrast, a fraction of people of African descent carry a functional allele because of a T<sup>125</sup>C SNP (nucleotide substitution c.373C>T) (24). To assess whether the expression of human caspase-12 plays a role in modulating metabolic inflammation, we examined African American obese children of the Yale Pediatric Obesity cohort for inflammatory markers in both the serum and the s.c. adipose tissue. ELISA measurements revealed decreased levels of CRP (p = 0.04) and IL-6 (p = 0.002) in the serum of carriers of the function C allele. This was most significant in boys (CRP: p = 0.02, IL-6: p = 0.006) compared with girls (CRP: p = 0.95, IL-6: p = 0.11) (Fig. 6A–F). Consistently, quantitative PCR (qPCR) analysis of eFAT from HFD-fed mice (n = 5 per genotype).
immunohistochemistry using a CD68-Ab (Fig. 6G–I). Next, to determine whether the CASP12 polymorphism is associated with metabolic disease parameters, we examined the African American cohort of the DHS (25). Association of the c.373T>C SNP with BMI, fasting blood glucose, HOMA-IR, hepatic TGs, and serum TGs revealed no protective effect in carriers of the C allele (Table I). Together, these results suggest that, in humans, loss of caspase-12 may not lead to obesity, in contrast with the effects observed in mice, but may contribute to inflammation associated with metabolic disease.

Discussion
It is now well-known that inflammation and metabolism are tightly interwoven processes, and fluctuations in one can have detrimental consequences in the other, potentially leading to disease. Studies in mice and humans have implicated various inflammatory pathways, including caspase-1 and the inflammasome, to obesity and diabetes (33). In this study, we investigated the role of the two related inflammatory caspases, caspase-11 and caspase-12, in a DIO mouse model. Interestingly, two mouse strains deficient in caspase-12 developed obesity on an HFD, whereas Casp11<sup>−/−</sup> or Ice<sup>−/−</sup> mice did not. The increased weight gain and adiposity of caspase-12–deficient mice was also associated with glucose intolerance and insulin resistance. Because Casp11<sup>−/−</sup> mice were equivalent to WT mice, deficiency in caspase-11 is unlikely to mediate the phenotype of Casp12<sup>−/−</sup> mice. This was confirmed in Casp12<sup>−/−</sup> mice that are sufficient for caspase-11. Caspase-11 is known to recognize intracellular LPS and intracellular bacterial pathogens (34). Obesity has been linked to increased gut permeability, resulting in elevated levels of

FIGURE 5. Casp12<sup>−/−</sup>Nlrp3<sup>−/−</sup> mice are protected from obesity and glucose intolerance, Casp12<sup>−/−</sup>L129<sup>−/−</sup> mice were crossed with Ripk2<sup>−/−</sup> or Nlrp3<sup>−/−</sup> mice to generate DKO strains Casp12<sup>−/−</sup>Nlrp3<sup>−/−</sup> and Casp12<sup>−/−</sup>Ripk2<sup>−/−</sup>. (A) Body-weight curve of male mice fed an LFD. (B) Relative weight of adipose depots and liver at 26 wk of age. Statistical analysis was performed using Student t test. Statistical significance is presented as follows: *p < 0.05, **p < 0.01, ***p < 0.001 versus the Casp12<sup>−/−</sup> group. (C) H&E staining of liver tissue of 26-wk-old mice. Scale bar, 200 μM. (D) Hepatic TGs and serum ALT. (E) Serum cholesterol. (F) Body-weight curve of male mice fed an HFD. (G) GTT of HFD mice at 22 wk of age injected i.p. with 2.0 mg/g dextrose. Error bars represent ± SEM. Determined by Student t test (*p < 0.05, **p < 0.01, ***p < 0.001 versus WT).
circulating microbial products and LPS (35). Although studies have linked numerous PRRs that detect bacterial motifs, such as Nod1/Nod2 (36) and TLR4 (37), to metabolic disease, caspase-11 may not play a similar role. However, because we observed slight differences between the two Caspase-12–deficient strains, we cannot rule out that Caspase-11 may become involved in the absence of Caspase-12. It has been observed that loss of Caspase-11 impacts *Salmonella* infection only in the absence of Caspase-1 (38), and it is possible that Caspase-12 deficiency could result in a small, Caspase-11–dependent effect in our model.

In contrast with males, female *Casp12<sup>−/−</sup>* mice were found to be equivalent in weight to WT mice when fed an LFD. Female mice are known to be protected from obesity because of the inhibitory role of estrogen on adipogenesis (39), and it is possible that the increased weight gain driven by loss of Caspase-12 could not compensate for this effect.

Caspase-12 has previously been demonstrated to have anti-inflammatory properties. Although the protease is catalytically active, currently its only known substrate is itself (21), and autoproteolysis was determined to not be required for its ability to inhibit both caspase-1 and NF-κB activity (16, 18). This suggests that caspase-12 primarily functions through modulating various signaling pathways through its ability to form CARD-CARD interactions. These include binding to caspase-1 (18), Ripk2 (17), NF-κB (16), and RIG-I (19), sterically hindering protein interactions and leading to attenuation of inflammation. Concordant with these functions, in our DIO mice we observed increased inflammation in the visceral adipose tissue, characterized by elevated inflammasome activation and immune cell infiltration. However, it is difficult to determine whether the increased inflammation is a consequence of obesity, rather than an underlying cause. Within the adipose tissue, Caspase-12 was not detectable in the radiosensitive compartment and is likely absent in infiltrating myeloid cells. Therefore the robust immune cell–driven inflammation observed in the adipose tissue at later stages of obesity may be independent of Caspase-12 activity.

It is unknown how caspase-12 may be affecting host metabolism, leading to the obese state of the mice. Indirect calorimetry and food intake measurements revealed no influence conferred by caspase-12 ablation. However, the obesity phenotype was dependent on loss of caspase-12 in the radio-resistant compartment. Caspase-12 is

![Image of Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Human caspase-12 dampens metabolic inflammation. Subjects carrying the risk allele for the T<sup>125</sup>C SNP showed lower CRP (A–C) and IL-6 (D–F) levels than the noncarriers. This phenomenon seemed to be more marked in males than in females. In the subgroup of subjects who underwent an adipose tissue biopsy, the T<sup>125</sup>C allele was associated with a lower percent of macrophages by immunohistochemistry and a lower expression of CD68, IL-6, and TNF-α by qPCR (G–I).
expressed in the muscle, liver, and fat (data not shown), tissues that are highly associated with metabolic health. Tissue-specific knockouts are required to determine precisely where the protease is required to suppress obesity. Interestingly, when Nlrp3, but not Ripk2, was deleted in the caspase-12 null background, the mice were protected from increased adiposity and weight gain. The Nlrp3 inflammasome and caspase-1 have been previously linked to the regulation of metabolic health in humans and in animal models, although there is some discrepancy in how it may regulate obesity in rodents. Caspase-1-, Nlrp3-, and ASC-deficient mice have been reported to be leaner than WT controls (11, 12), equivalent in body weight (7, 8), or have increased adiposity (14, 15, 40). Obesity in these mice is also correlated with reduced glucose tolerance, suggesting that any beneficial effects on metabolic health that loss of the inflammasome may have are not significant enough to counteract other pathways influenced by obesity. Differences in adipogenesis (11), cleavage of SIRT1 (41), and alteration of lipid metabolism (13, 15) are a few of the proposed mechanisms by which caspase-1 may regulate metabolic processes. In the context of obesity, caspase-12 might impact caspase-1 directly or indirectly. Loss of Nlrp3 may antagonize direct effects of caspase-12 on caspase-1, conferring a lean phenotype. Nlrp3 and Caspase-12 are both expressed in adipocytes (42) (Supplemental Figs. 1A, 3D), which represents one potential radio-resistant cell type where an interaction between the two proteins may take place. Alternatively, caspase-12 may affect a parallel metabolic pathway that is reversed by loss of Nlrp3. For example, the inflammasome has been linked to gut microbiota dysbiosis, which affects obesity (14). Potential microbial dysbiosis in caspase-12–deficient mice may be altered upon removal of Nlrp3. Further work is required to determine how caspase-12 may function in radio-resistant cells, and the use of tissue-specific knockouts may aid in examining how these potential interactions may drive metabolic health in mice.

Although the majority of people lack a functional caspase-12, the T^125C SNP has persisted in a small percent of people of African descent. Many studies have examined why the functional caspase-12 allele has been maintained, including examining a potential role in protection against bacterial sepsis (24, 43), candidemia (44), and rheumatoid arthritis (45). Our previous work has implicated that the T^125C SNP may confer susceptibility to sepsis (24); however, a second study assessing community-acquired pneumonia did not report similar findings (43). There is no clear indication what role caspase-12 may play in people and given how these potential interactions may drive metabolic health in mice.

Table 1. Demographic, anthropometric, and clinical characteristics of African American participants of the DHS stratified by sex and CASP12 R125X (rs97116) genotype

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Male (n=1042)</th>
<th>Female (n=1412)</th>
<th>Total (n=2454)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>40.0 ± 11</td>
<td>41.0 ± 11</td>
<td>40.0 ± 11</td>
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<tr>
<td>BMI, kg/m²</td>
<td>29.2 ± 6.9</td>
<td>28.6 ± 7.7</td>
<td>29.0 ± 7.1</td>
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<tr>
<td>Glucose, mg/dl</td>
<td>92.0 ± 5.0</td>
<td>91.0 ± 5.5</td>
<td>91.0 ± 5.5</td>
</tr>
<tr>
<td>TG, mg/dl</td>
<td>5.7 ± 11</td>
<td>5.7 ± 11</td>
<td>5.7 ± 11</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>3.3 ± 2.3</td>
<td>3.3 ± 2.3</td>
<td>3.3 ± 2.3</td>
</tr>
</tbody>
</table>

Analysis of T^125C SNP in African American cohort of DHS. The DHS population was genotyped for the Caspase-12 T125C SNP using the Illumina Human Exome chip, as described in Materials and Methods. The analysis was performed using linear regression, adjusted for age and BMI and stratified by sex. Values are mean ± SEM. "n" parentheses indicate the number of individuals with available data for each phenotype.

**Table 2.** Summary of findings in mice. The Nlrp3 inflammasome and caspase-1 have been previously linked to the regulation of metabolic health in humans and in animal models, although there is some discrepancy in how it may regulate obesity in rodents. Caspase-1–, Nlrp3–, and ASC-deficient mice have been reported to be leaner than WT controls (11, 12), equivalent in body weight (7, 8), or have increased adiposity (14, 15, 40). Obesity in these mice is also correlated with reduced glucose tolerance, suggesting that any beneficial effects on metabolic health that loss of the inflammasome may have are not significant enough to counteract other pathways influenced by obesity. Differences in adipogenesis (11), cleavage of SIRT1 (41), and alteration of lipid metabolism (13, 15) are a few of the proposed mechanisms by which caspase-1 may regulate metabolic processes. In the context of obesity, caspase-12 might impact caspase-1 directly or indirectly. Loss of Nlrp3 may antagonize direct effects of caspase-12 on caspase-1, conferring a lean phenotype. Nlrp3 and Caspase-12 are both expressed in adipocytes (42) (Supplemental Figs. 1A, 3D), which represents one potential radio-resistant cell type where an interaction between the two proteins may take place. Alternatively, caspase-12 may affect a parallel metabolic pathway that is reversed by loss of Nlrp3. For example, the inflammasome has been linked to gut microbiota dysbiosis, which affects obesity (14). Potential microbial dysbiosis in caspase-12–deficient mice may be altered upon removal of Nlrp3. Further work is required to determine how caspase-12 may function in radio-resistant cells, and the use of tissue-specific knockouts may aid in examining how these potential interactions may drive metabolic health in mice.

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Disclosures
The authors have no financial conflicts of interest.

References