Inactivation of C/ebp Homologous Protein-driven Immune-Metabolic Interactions Exacerbate Obesity and Adipose Tissue Leukocytosis*

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Background: C/ebp homologous protein contributes to ER stress-induced inflammatory cytokine secretion and apoptosis. Loss of Chop increases obesity, insulin resistance, and adipose tissue leukocytosis and inflammation. ER stress signaling through Chop is necessary for adaptation to a high fat diet. Although in obesity ER stress may impair metabolic health, inactivation of Chop promotes obesity-associated inflammation.

Successful adaptation to periods of chronic caloric excess is a highly coordinated event that is critical to the survival and propagation of species. Transcription factor C/ebp homologous protein (Chop) is thought to be an important molecular mediator that integrates nutrient signals to endoplasmic reticulum (ER) stress and innate immune activation. Given that aberrant ER stress response is implicated in inducing metabolic inflammation and insulin resistance, we hypothesized that ER stress target gene Chop integrates immune and metabolic systems to adapt to chronic positive energy balance. Here we report that inactivation of Chop in mice fed a high fat diet led to significant increase in obesity caused by a reduction in energy expenditure without any change in food intake. Importantly, ablation of Chop does not induce metabolically healthy obesity, because Chop-deficient mice fed a high fat diet had increased hepatic steatosis with significantly higher insulin resistance. Quantification of adipose tissue leukocytosis revealed that elimination of Chop during obesity led to substantial increase in number of adipose tissue T and B lymphocytes. In addition, deficiency of Chop led to increase in total number of myeloid subpopulations like neutrophils and F4/80⁺ adipose tissue macrophages without any alterations in the frequency of M1- or M2-like adipose tissue macrophages. Further investigation of inflammatory mechanisms revealed that ablation of Chop increases the sensitivity of macrophages to inflammasome-induced activation of IL-β in macrophages. Our findings indicate that regulated expression of Chop during obesity is critical for adaptation to chronic caloric excess and maintenance of energy homeostasis via integration of metabolic and immune systems.

The endoplasmic reticulum (ER) stress response maintains cellular homeostasis during times of ER dysfunction. ER stress is sensed by three main proteins: X-box binding protein 1, PRKR-like endoplasmic reticulum kinase (also known as eukaryotic translation initiation factor 2-α kinase 3), and activating transcription factor-6, which cooperate to mitigate ER stress by reducing protein translation, stabilization of proteins by chaperones, and activating ER associated protein degradation (1). However, when either the magnitude or duration of ER stress goes beyond these coping mechanisms, apoptosis occurs (2). The PRKR-like endoplasmic reticulum kinase elongation initiation factor 2α-activating transcription factor 4 signaling pathway connects ER stress to Chop (C/ebp homologous protein) and contributes to ER stress-induced apoptosis (1–3). Chop exerts its apoptotic effects by altering transcription of BCL-2 family proteins and also causing release of calcium from the ER (2–4).

Obesity is a state of increased ER stress, and ER stress contributes to the immunometabolic dysregulation that leads to low grade systemic inflammation and the development of metabolic syndrome (5, 6). Chemical chaperones that mitigate ER stress (4-phenylbutyrate and tauroursodeoxycholic acid) through protein stabilization improve systemic glucose homeostasis, increase glucose uptake in adipose and skeletal muscle, and reduce hepatic glucose production (6). In humans, weight loss reduces ER stress and treatment of obese subjects with tauroursodeoxycholic acid improves both skeletal muscle and liver insulin sensitivity (7, 8). Thus, targeting ER stress may have therapeutic value for the management of obesity and its co-morbidities.

Disruption of Chop signaling leads to metabolic improvements in models of diabetes (9). In models of pancreatic beta cell dysfunction, Chop ablation improves glucose homeostasis and reduces beta cell death (9). Chop ablation also appears to
impact adipose tissue. In adipose tissue, Chop acts as an inhibitor of adipogenesis in the 3T3-L1 adipocyte cell line (10). Male and female Chop−/− mice fed a high fat diet have normal glucose tolerance despite having increased fat mass and hepatic steatosis (11). Thus, the Chop deficiency appears to protect against obesity-induced metabolic impairment; however, it is not known how it impacts adipose tissue inflammation.

During obesity, adipose tissue acquires enhanced immunological properties, including increased infiltration of hematopoietic lineage cells, such as activated macrophages, neutrophils, and T and B cells that cause increased inflammation and insulin resistance (12, 13). In macrophages, ER stress leads to activation of the NLRP3 (NLR family pyrin domain containing 3) inflammasome, a large cytosolic multiprotein scaffolding complex that activates caspase-1 and leads to the secretion of bioactive IL-1β (14). Inflammasome activation has been shown to impair insulin sensitivity in adipose tissue, liver, and skeletal muscle and increases adipose tissue inflammation (15–17). Interestingly, Chop amplifies IL-1β secretion through its role in release of calcium from the ER and also increases macrophage apoptosis in response to lipotoxicity (18, 19). Thus, Chop-mediated immunometabolic cross-talk during obesity could be a potential target to ameliorate obesity-associated comorbidities (10). Surprisingly, we found that Chop ablation led to increased obesity, hepatic steatosis, impaired glucose, and insulin homeostasis. These metabolic impairments were accompanied by inflammasome activation and leukocytosis of adipose tissue.

MATERIALS AND METHODS

Animal Care—Chop−/− (B6.129S(Cg)-Ddit3tm2.1Dron/J) mice were obtained from Jackson Laboratory and have been previously described (20). The male littermate WT (C57/BL6) and Chop−/− mice were placed on a 60% high fat diet (D12492; Research Diets, Inc., New Brunswick, NJ) at 6–8 weeks of age and sacrificed at 5 months of age. The mice were housed at 22 °C in the specific-pathogen free barrier facility in ventilated cage racks that delivers HEPA-filtered air to each cage with free access to sterile water (without hyperchloration) through a hydropac system. The sterile water-containing Hydropacs are changed weekly to avoid development of microbial biofilms that can cause alteration in gut microflora. In addition, sentinel mice in our animal rooms were negative for currently tested standard murine pathogens (Ectromelia, EDIM, LCMV, Mycoplasma pulmonis, MHV, MNV, MPV, MVM, PVM, REO3, TMEV, and Sendai virus) at various times while these studies were performed (RADIL; Research Animal Diagnostic Laboratory, Columbia, MO). All experiments and animal use were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Pennington Biomedical Research Center. All tissues were flash frozen in liquid nitrogen.

Body Composition and Energy Expenditure—Body composition was assessed by NMR (Minispec LF50 TD-NMR system; Bruker Optics, Billerica, MA) to determine fat mass, fat free mass, and body fluid. The PhenoMaster/LabMaster (TSE Systems, Bad Homburg, Germany), which assesses O2 consumption and CO2 production, food and water intake, and physical activity, was used to assess food intake, energy expenditure, respiratory exchange rate, and activity. O2 and CO2 measurements were taken every 25 min during a 72-h period.

Glucose and Insulin Tolerance Tests—Mice were fasted for either 12 h (glucose tolerance test) or 4 h (insulin tolerance test). Mice received an intraperitoneal injection of either insulin (0.08 milliunit/g of body weight; Sigma-Aldrich) or glucose (0.4 mg/g; Sigma-Aldrich). Tail vein blood glucose was assessed using a glucometer (Breeze; Bayer Health Care, Tarrytown, NY) at baseline 0, 10, 20, 30, 45, 60, and 90 min.

Blood Analysis—Triglycerides and total cholesterol were measured by Cardiocheck lipid panel (Cardiocheck, Indianapolis, IN) in whole blood. To obtain serum, whole blood was incubated at room temperature for 2 h and the centrifuged at 3,000 rpm for 20 min. Serum was analyzed for hormones and cytokines using a multiplex bead assay (Millipore, Billerica, MA).

Adipose Tissue Preparation—Epididymal adipose tissue was harvested, weighed, and then minced in 0.01% type I collagenase. After mincing, cells were incubated for 1 h in a shaking water bath (37 °C) and vortexed every 10 min. Samples were then centrifuged at 1,500 rpm at 4 °C for 5 min. The supernatant was discarded, and the stromal vascular pellet was ACK lysed to remove red blood cells and filtered through a 100-μm cell strainer. Another centrifugation step was performed, and the pellet was washed in 5 ml of PBS. The stromal vascular fraction (SVF) was then filtered again through a 40-μm cell strainer and centrifuged. The SVF cell pellet was resuspended in complete RPMI and counted.

Flow Cytometry—Thymic, splenic, and visceral adipose tissue (VAT) SVF cells were stained in stain buffer with FBS (BD Pharmingen), and anti-CD16/CD32 (eBioscience) was used to block Fc receptors. To assess macrophage, granulocyte, B cell, and T cell populations, cells were stained with anti-F4/80, CD11c, CD206, Gr-1, B220, CD3, CD4, CD8, CD44, and CD62L (eBiosciences and Biolegend). A FACSCalibur (BD Pharmingen) was used for flow cytometry analysis, and all the

| Symbol | Primer | Sequence (5′ → 3′) |
|--------|--------|-----------------|
| Arg1   | Forward | ATTACTGGAGCGCTCTTTCTCC |
| Atf6   | Reverse | TTTTCCTCAAGACTGTTTCTT |
| Bcl2   | Forward | GCCCTCTGTCCTTGCTCCTG |
| Casp3  | Reverse | AGTGGCTCTACGAGAGGG |
| Casp8  | Forward | ACCAGGCTCAAGAGACTTGC |
| Ccl3   | Reverse | TTGGACCTTCCGCTGTGAG |
| CCL4   | Reverse | CATTAGGCTTCCGCTGTGAG |
| Foxp3  | Forward | CGGCAACACCCAGCCCTAGT |
| Gapdh  | Reverse | CCATTCCAGACTGCGCATC |
| IL-10  | Forward | TCTCCTGATGTCCTCATGAG |
| iNos   | Reverse | AGCTACTTCCCTGCTGTGAG |
| Xbp1   | Forward | GAGTCGGCCATCCTCAACCTT |

TABLE 1
FACS data were compensated using FlowJO (Treestar Inc.) software.

**Adipose Tissue and Liver Histology**—Tissues were formalin-fixed (4%) and paraffin-embedded. After embedding, sections were stained with hematoxylin and eosin. Images were acquired using a Nanozoomer (Hamamatsu Corporation, Bridgewater, NJ).

**Cell Culture**—All steps were performed using sterile technique. Femurs were collected in RPMI (Invitrogen) + 5% FBS (R5; Omega Scientific, Tarzana, CA). Both ends of the femur were then cut, and the femur was flushed with R5. The bone marrow was centrifuged at 450 × g for 5 min, the supernatant was decanted, and red blood cells were lysed using ACK lysis buffer (Quality Biological, Gaithersburg, MD). After neutralization with R5, bone marrow cells were centrifuged, resuspended in 10 ml of R5, and placed into a 6-well plate. Nonadherent cells were collected the following morning. The nonadherent cells were resuspending at 4 × 10^6 cells/ml in medium consisting of 10 ml supernatant of nonadherent cells, 7.2 ml of L929 conditioned media, 6.8 ml of R5, and MCSF (10 ng/ml; R&D Systems, Minneapolis, MN). An additional 2 ml of fresh medium was added 4 days after isolation. Nonadherent cells were collected on day 7 and separated by density gradient separation using Fico/Lite (Atlanta Biologicals, Flowery Branch, GA), and mononuclear cells were collected. The cells were rinsed twice with Dulbecco’s PBS + 2% FBS and resuspended at 1 × 10^6 cells/ml. The cells were treated with ultrapure LPS (Invivogen, San Diego, CA) alone or in combination with 5 mM ATP (Sigma) or 200 μM palmitate-BSA (Sigma).

**Western Blotting**—Adipose tissue was disrupted using liquid nitrogen and a mortar and pestle. The tissue was then resuspended in RIPA supplemented with protease and phosphatase inhibitors and further processed using a pellet pestle. Samples were left on ice for 1 h with vortexing every 10 min. Samples were resuspending at 4 × 10^6 cells/ml in medium consisting of 10 ml supernatant of nonadherent cells, 7.2 ml of L929 conditioned media, 6.8 ml of R5, and MCSF (10 ng/ml; R&D Systems, Minneapolis, MN). An additional 2 ml of fresh medium was added 4 days after isolation. Nonadherent cells were collected on day 7 and separated by density gradient separation using Fico/Lite (Atlanta Biologicals, Flowery Branch, GA), and mononuclear cells were collected. The cells were rinsed twice with Dulbecco’s PBS + 2% FBS and resuspended at 1 × 10^6 cells/ml. The cells were treated with ultrapure LPS (Invivogen, San Diego, CA) alone or in combination with 5 mM ATP (Sigma) or 200 μM palmitate-BSA (Sigma).
were then centrifuged at 14,000 × g for 15 min, the supernatant was collected, and the protein concentration was determined using the DC protein assay (Bio-Rad). Analysis of AKT and IL-1β was performed as described previously (15). Gene Expression Analysis—Total RNA was extracted using the TRIzol method, transferred to the Qiagen RNeasy mini kit, and purified according to the manufacturer’s instructions. On column DNA digestion was performed to remove DNA. Synthesis of cDNA and quantitative PCR was performed as described previously (15). Primer sequences for Arg1, Atf6, Bcl2, Casp3, Casp8, Casp9, Ccl3, Ccl4, Foxp3, Gapdh, IL-10, iNos, and XBP1 are listed in Table 1.

Statistics: Energy Expenditure/Other—Energy expenditure was calculated using indirect calorimetry. The last 168 h of measurements were used to calculate total 24-h energy expenditure (total energy expenditure (TEE); expressed in kcal/day) and respiratory exchange ratio (VCO2/VO2). Resting energy expenditure (REE; expressed in kcal/day) was calculated by the lowest 1-h period of energy expenditure and then extrapolated to 24 h. This happens to coincide with the lowest 1 h of ambulatory activity. TEE was related to both fat-free mass (FFM) and fat mass (FM) by multiple regression analysis (TEE = −5.89 + 0.82 × FFM + 0.03 × FM; R² = 0.84, p < 0.0001). This multiple-regression equation was used to predict TEE for all mice. The residuals (i.e., the differences between measured and predicted values) were then calculated for each animal and were tested against the wild type regressions that should equal 0. Multiple regression equations relating REE to FFM and FM were used to predict REE values, and residuals were subsequently calculated using the same method described above (REE = −4.8 + 0.58 × FFM + 0.13 × FM; R² = 0.85, p < 0.0001). Data analysis was performed using JMP Pro 10 statistical discovery software (SAS Institute, Cary, NC). Student’s t test for paired samples was used to assess differences with significance defined as p < 0.05.

RESULTS
Deletion of Chop Increases Obesity—Chop is an important mediator of ER stress and is thought to play a role in weight gain and adiposity (10). It has previously been suggested that deletion of Chop in mice increases body weight, and this was asso-

FIGURE 2. Diet-induced obese Chop−/− mice develop increased insulin resistance and hepatic steatosis. A and B, glucose (A) and insulin tolerance (B) in 4-month HFD-WT and Chop−/− mice. C, the immunoblot analysis of pAKT and tAKT in visceral adipose tissue 5 min postinsulin injection with ratio of band density of pAKT normalized to tAKT in 5-month HFD-fed WT and Chop−/− mice. D, representative image of hematoxylin- and eosin-stained liver section of WT and Chop−/− mice fed HFD for 5 months. E–H, total serum cholesterol (E), triglycerides (F), leptin (G), and total PAI-1 (H) of 5-month WT and Chop-deficient mice. Significance is designated by * (p < 0.05) and # (p < 0.10); (n = 8–16/group).
FIGURE 3. Chop does not control T cell development or homeostasis in lymphoid organs of obese mice. A–D, frequency of thymic double positive (A), CD8 single positive (B), CD4 single positive (C), and CD4 CD8 double negative (D, DN) populations in WT and Chop−/− mice fed HFD for 5 months (n = 8/group). E, representative FACS plots of splenocytes isolated from 5-month HFD-fed WT and Chop−/− mice. E and F, the dot plot depicts (E) CD4+ and (F) CD8+ T cells subpopulation; naïve (CD44+CD62L−), effector-memory population (CD44+CD62L−), and central memory (CD44+CD62L−) populations. G and H, the quantification of frequencies of CD4 (G) and CD8 (H) T cell subpopulations in spleens derived from 5-month-old DIO WT and Chop-deficient mice (n = 8/group).

FIGURE 4. Inactivation of Chop during obesity enhances myeloid cell recruitment in VAT. A and B, representative FACS dot plots showing sequential gating of SVF cells isolated from VAT of 5-month HFD-fed WT (A) and Chop−/− (B) mice. The left dot plot depicts FSC and SSC, the sequential gating for F4/80+ macrophages, and pro-inflammatory M1-like (F4/80+CD11c−CD206−) and alternatively activated M2-like (F4/80+CD11c+CD206+) macrophages. The frequency of target cell population is provided for each gate. C–E, the total number of F4/80+ cells (C), M1-like F4/80+CD11c−CD206− (D), and M2-like F4/80+CD11c+CD206+ (E) adipose tissue macrophages/g of visceral adipose tissue. F, ratio of M1-like/M2-like macrophages. G, total Gr1+ F4/80+ granulocytes/g of visceral adipose tissue. Significance is designated by * (p < 0.05; n = 8/group).
C/ebp Homologous Protein Loss Increases Adipose Inflammation

(A) WT (Visceral fat)

(B) Chop/− (Visceral fat)

(C) L1 gated

(D) L1 gated

(E) L1 gated

(F) L2 gated

(G) L2 gated

(H) L2 gated

Lymphocytes/g tissue

B220+ cells/g tissue

CD3+ cells/g tissue

Lymphocytes/g tissue

B220+ cells/g tissue

CD3+ cells/g tissue
associated with either improved glucose homeostasis (9) or maintenance of insulin sensitivity (10, 11). Thus, the role of Chop in control of energy homeostasis is not established. Therefore, our initial studies investigated whether deletion of Chop exerts protective role in obesity and whether this is due to changes in energy intake or energy expenditure. Compared with high fat diet-fed WT littermate controls, Chop−/− mice had increased body weight, FM, FFM, and body fluid (Fig. 1A). We next sought to determine whether altered food intake or energy expenditure explained increased body weight in Chop−/− mice. Compared with HFD-fed WT, Chop−/− mice had a trend toward reduced TEE when controlled for FM, FFM, and activity (distance moved/day) and significantly reduced REE (Fig. 1B). Compared with WT mice, the Chop−/− mice fed HFD did not show any significant difference in food intake (Fig. 1C). The difference in TEE is biologically important because the kcal accounted for by reduced total energy expenditure (~1.5 kcal/day) would contribute to a weight gain of ~1 g/week and may account for the differences in body weight noted between Chop−/− and WT mice. Notably, there was also a trend toward reduced activity (Fig. 1D) in HFD-Chop−/− compared with WT mice. No differences in food intake, respiratory exchange ratio, or its variation over 24 h were noted (Fig. 1, E and F).

**Chop Ablation Impairs Glucose Homeostasis and Leads to Hepatic Steatosis—**Given that prior work shows that loss of Chop function improves glucose homeostasis, we next evaluated whether inactivation of Chop may induce metabolically healthy obesity. Surprisingly, Chop−/− mice exhibited a high degree of glucose intolerance compared with WT mice (Fig. 2A). Importantly, two cohorts of mice (n = 7–8/genotype in each group; data shown from second cohort) were necessary for glucose tolerance testing because 50% of the first cohort of Chop−/− mice had glucose values above the glomerulectomy detection limit for an extended period of time, suggesting significantly worse insulin resistance upon inactivation of Chop. Chop−/− mice also displayed impaired insulin sensitivity (Fig. 2B) in an insulin tolerance test. Furthermore, compared with HFD WT mice, adipose tissue of Chop−/− mice had reduced insulin-stimulated phosphorylation of AKT (Fig. 2C), suggesting impaired insulin signaling. Upon dissection of DIO Chop−/− mice, the livers were noticeably yellow and steatotic, which was confirmed by hematoxylin and eosin staining of liver sections (Fig. 2D). Consistent with the development of hepatic steatosis, total cholesterol was increased in Chop−/− mice compared with WT mice; however, triglycerides were unaffected (Fig. 2, E and F). Unexpectedly, serum leptin was not increased in HFD Chop−/− compared with WT mice, as would be expected with the increased adipose tissue mass of Chop-deficient animals (Fig. 2G). Total PAI-1, a hepatocyte-derived acute phase protein that contributes to chronic low grade inflammation and obesity-associated vascular disease, had a trend (p = 0.08) toward being increased in Chop−/− mice (Fig. 2H).

**Lymphoid Organ T Cell Homeostasis Is Not Altered by Chop Ablation during Obesity—**Given that changes in immune cell homeostasis can influence energy metabolism and vice versa (12, 13, 21), we investigated the T cell subpopulations in thymus and spleen. The CD4+ CD8− (double positive), CD4 single positive (CD4SP), CD4− CD8− double negative T cells populations were not different between DIO Chop−/− and WT mice (Fig. 3, A–D); however, there was a trend toward reduced CD8 single positive (CD8SP) cells in the thymus of Chop−/− mice (Fig. 3B). Consistent with these findings, CD4+ and CD8− naïve and effector populations in the spleen were unaffected by Chop deletion during obesity (Fig. 3, E and F), and there were no alterations in the splenic CD4+ and CD8− naïve (CD44− CD62L+) and effector memory (CD44+ CD62L−) cell populations (Fig. 3, E–H). These results indicate that Chop-mediated metabolic alterations during obesity are not sufficient to impact the systemic T cell homeostasis in thymus and spleen.

**Inactivation of Chop Leads to Adipose Tissue Leukocytosis during Obesity—**We next sought to understand the role of Chop in immune-metabolic interactions within VAT. The flow cytometric analysis of the SVF of VAT revealed that ablation of Chop during obesity led to a significant increase in hematopoietic cells. Compared with HFD WT mice, inactivation of Chop produced a greater than 2-fold increase of F4/80+ cells and M1-like (F4/80+ CD11c+ CD206−) macrophages (Fig. 4, A–D). Interestingly, there was a greater than 4-fold increase in M2-like (F4/80+ CD11c− CD206+) macrophages (Fig. 4, A, B, and E). The ratio of M1/M2 cells in VAT was reduced in Chop−/− mice because of the increased number of M2 macrophages (Fig. 4F). Interestingly, the total numbers of Gr1+ F4/80+ granulocytes that represent mainly neutrophils were also elevated in HFD-fed Chop-deficient mice in comparison with the WT controls (Fig. 4G). These findings suggested that loss of Chop function enhances overall myeloid cell infiltration within the VAT.

**Chop Controls Adipose Tissue Lymphocytosis in Obesity—**In addition to macrophages and granulocytes, both T and B cell numbers were increased with Chop ablation during diet-induced obesity (Fig. 5A). Interestingly, unlike lymphoid organs, ablation of Chop increased the T cell frequency in VAT but not the B220 cell percentages (Fig. 5, A and B). Interestingly, obese Chop−/− mice displayed a unique population of VAT T cells (L2 gate) that were larger in size (Fig. 5B). The larger size lymphocytes in lymphoid organs typically represent activated cells. The number of regular size lymphocytes (in L1 gate), B220+ B cells, and CD3+ T cells/g tissue in the smaller gate was approximately two times greater in Chop−/− mice (Fig. 5, C–E). The activated T cells/g tissue (L2 gated cells) were approximately three times greater in HFD-fed Chop−/− compared with WT mice (Fig. 5, F and H). The activated B cell population in L2 gate was not significantly different between WT and Chop-deficient obese mice (Fig. 5G). These results indicate an increased infiltration of lymphoid cells into VAT during obesity in the

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**FIGURE 5. Deletion of Chop induces adipose tissue lymphocytosis during obesity.** A and B, representative FACS dot plots of SVF cells isolated from VAT of 5-month HFD-fed (A) WT and (B) Chop−/− mice. A and B, the left dot plots depict FSC and SSC and gating for smaller lymphoid population (L1 gate) and a larger cell population (L2). The frequencies of each target cell population in arrows depict the sequential gates for B220+ and CD3+ lymphocytes. C–H, quantification of lymphocytes derived from VAT of DIO WT and Chop null mice. C, total number of lymphoid cells. D, B cells. E, T cells. F, T cells within the L1 gate. G, the quantification of lymphocytes (cell number/g of fat) in larger lymphoid population (L2 gated) within VAT of WT and Chop−/− mice fed HFD for 5 months. Significance is designated by * (p < 0.05; n = 8/group).
absence of Chop signaling. In addition, the absence of Chop during obesity leads to emergence of a T and B cell subpopulation (in L2 gate) that is larger in size indicating their potentially higher activation status.

Elimination of Chop during Obesity Sensitizes the Macrophages to Inflammasome Activation—Consistent with our data demonstrating increased VAT leukocytosis, the hematoxylin and eosin staining revealed that visceral adipose tissue of obese Chop<sup>−/−</sup> mice had a large infiltration of hematopoietic cells and an increase in crown-like structures compared with WT mice (Fig. 6, A and B). Chop has previously been shown to amplify inflammasome signaling (18); however, contrary to prior studies, we found that elimination of Chop signaling during obesity led to increased expression of pro-IL-1β (Fig. 6C).

**FIGURE 6.** Ablation of Chop promotes inflammasome activation and VAT inflammation during obesity. A and B, visceral adipose tissue section of HFD-WT (A) and Chop<sup>−/−</sup> (B) mice stained with hematoxylin and eosin. C, Immunoblot analysis of IL-1β and in VAT of WT Chop<sup>−/−</sup> mice. D, the bone marrow-derived macrophages from WT and Chop-deficient mice were primed with ultrapure LPS for 4 h and stimulated with Nlrp3 inflammasome activators ATP (4 mM) and palmitate (200 μM). The BMDM supernatant were used for immunoblot analysis of activated IL-1β (p17) WT and Chop<sup>−/−</sup> mice (n = 3). E, real time PCR analysis of Foxp3, iNos, Arg1, Il10, Ccl3, Ccl4, Atf6, Xbp1, Casp3, Casp8, Casp9, and Bcl2 in SVF cells of WT and Chop<sup>−/−</sup> mice fed a high fat diet for 5 months. Gene expression was normalized to Gapdh. Significance is designated by * (p < 0.05; n = 5–8/group). Con, control.
that may reflect increased number of adipose tissue macrophages and neutrophils in VAT. The circulating and tissue-activated p17 IL-1β was below the assay sensitivity. In addition, ablation of Chop did not affect circulating IL-18 levels (data not shown). Because of our findings of increased adipose tissue leukocytosis and pro-IL-1β protein levels, we next sought to determine the influence of Chop on inflammasome activation. Interestingly, compared with BMDMs derived from WT mice, the Chop-deficient BMDMs that were primed with lipopolysaccharide and exposed to the Nlrp3 inflammasome inducers extracellular ATP and lipotopic fatty acid palmitate displayed an increase in activated p17 IL-1β (Fig. 6D). Gene expression analysis of SVF indicates a loss of regulatory T cells, with reduced expression of Foxp3 in HFD Chop-deficient compared with WT mice (Fig. 6E). The SVF of Chop-deficient mice also had increased expression of both M1-like and M2-like marker iNOS and Arg1, with no change in anti-inflammatory cytokine IL10, which is also associated with regulatory B cells. In addition, chemokines CCL3 and CCL4 were not significantly in WT and mutant mice (Fig. 6E). There was no difference in SVF gene expression of Atf6 or Xbp1, indicating that these pathways were up-regulated to compensate for absence of Chop (Fig. 6E). There was increased expression of the proapoptotic gene Caspase3 in DIO Chop-deficient mice; however, the other apoptotic genes Caspase8 and Caspase9 and pro-survival gene Bcl2 were not different (Fig. 6E). These data suggested that ablation of Chop alters the immune metabolic axis by increasing IL-1β production, reducing regulatory T cells, without binary changes in M1 or M2 macrophage associated cytokines.

**DISCUSSION**

The ER stress is considered to be an important regulator of immune-metabolic cross-talk during obesity. The alteration in UPR response and activation of ER stress regulator Chop has therefore been hypothesized to not only control inflammation and metabolic complications of obesity. In this report, we demonstrate that Chop-mediated signaling is required by the host for adaptation to chronic stress imposed by high fat diet-induced caloric excess.

The inactivation of Chop increases HFD-induced adipose tissue inflammation and impairs glucose and insulin tolerance. However, in models of type 2 diabetes, Chop signaling impairs glucose homeostasis by reducing pancreatic islet size and increasing beta cell death as part of its apoptotic program (22). Chop ablation improved insulin secretion and glucose tolerance in akita mice, db/db mice, streptozotocin-treated mice, and elf2αS/A mice that undergo beta cell failure and develop diabetes while being fed a HFD (9). Interestingly, in that study, after 5 weeks of HFD, Chop−/− mice had improved glucose tolerance, which is in contrast to our chronic HFD feeding experiment. It is known that during high fat feeding, adipose tissue remodeling follows a time course with the highest turnover of adipocytes occurring at ~16 weeks of feeding, which is also the time of greatest impairments of insulin sensitivity (23). Our histological and immunological analysis of Chop−/− mice indicates an increased remodeling of adipose tissue compared with WT mice. The resultant inflammation from this remodeling process appears to be overriding any beneficial effect of Chop on pancreatic function by impairing glucose homeostasis.

Factors that drive adipogenesis, such as PPARγ activators, improve insulin sensitivity and reduce adipose tissue inflamma-

 tion (24). Han et al. (10) found that ER stress signaling proteins are increased during adipogenesis, and ER stressors inhibit adipogenesis. Inhibition of EIF2α and induction of Chop production inhibited adipogenesis, which led Han et al. (10) to hypothesize that Chop deletion would have a beneficial effect on adipose tissue function through the promotion of adipogenesis. We found that although Chop−/− mice have increased adiposity, this was accompanied by a high degree of adipose tissue inflammation. Han et al. found no alterations in fasting blood glucose in WT and Chop−/− mice fed a 45% HFD for 13 weeks; however, no glucose or insulin tolerance tests were performed on these mice. Similar to the prior study, we also observed increased adiposity upon ablation of Chop; however, we observed striking impairment in blood glucose homeostasis. Surprisingly, obese Chop-deficient mice had a profound leukocytosis of adipose tissue, and this occurred in the absence of changes in circulating leptin. Thus, it appears that the proadiogenic effects of Chop ablation are overcome by the ensuing pro-inflammatory response by HFD that exacerbates metabolic syndrome.

The influence of Chop on inflammation is complex and may be dependent on specific animal models of immune cell activation. During lipopolysaccharide-induced lung injury, Chop knock-out animals are protected against inflammatory damage (25). However, during lipopolysaccharide-induced kidney injury, Chop deficiency exacerbates kidney inflammation and damage, similar to the results we found in adipose tissue (26). The reason for heterogeneity of tissue response to Chop ablation is unclear; yet this may explain the protective aspects of Chop ablation on the pancreatic beta cells and the detrimental effects of whole body Chop deletion in response to a HFD.

We found that during obesity, Chop ablation did not dampen inflammation in adipose tissue. Instead, Chop-deficient mice had increased crown-like structures, recruitment of macrophages, and other leukocytes, pro-IL-1β, and decreased expression of the regulatory T cell marker Foxp3 in adipose tissue while being fed a HFD. The NLRP3 inflammasome is activated by numerous danger-associated molecular patterns including ATP, saturated fatty acids, and ceramides (27). ER stress induces IL-1β secretion by the Nlrp3 inflammasome (14), and Chop acts as an amplifier in this process (18). Contrary to our findings, previous studies indicate that loss of Chop reduces caspase-1 and IL-1β activation, suggesting that Chop mediated responses serve as inflammasome amplifier (18). However, Chop ablation in BMDMs did not reduce IL-1β in response to ATP and palmitate, indicating that inactivation of Chop alters the cellular homeostasis by inducing inflammasome activation. Thus, the role of Chop in inflammasome activation in context of metabolic syndrome must be re-evaluated. Our data also indicate that adipose tissue inflammatory changes occur in the absence of increased apoptotic signals or changes in other ER stress pathways. We did note an increase in Casp3 mRNA; however, Casp3 cleavage is reduced in the absence of Chop (28). Thus, it appears that Chop plays a role in dampening inflamma-
tion during obesity, and new models will be needed to ascertain the cell specific effects of this response.

The ER stress response in the liver integrates short term changes in diet as well as chronic metabolic stressors. Interestingly, Chop^+/−^ animals do not have differences in hepatic steatosis during short term challenge with tunicamycin or longer term treatment with a methionine choline-deficient diet, which causes nonalcoholic fatty liver disease like changes in the liver (29). Although the methionine choline-deficient diet causes changes similar to nonalcoholic fatty liver disease, both WT and Chop^+/−^ mice on this diet are lean and maintain insulin sensitivity (19, 30). Recent data suggest that Chop promotes the resolution of liver inflammation through the promotion of Kupfer cell apoptosis, and disruption of this activity in Chop^−/−^ animals promotes liver inflammation and steatosis during high fat feeding and methionine choline-deficient diet (19). Our data show that high fat diet and impaired glucose and insulin tolerance is associated with hepatic steatosis in Chop^−/−^ mice. In addition, circulating levels of PAI-1, which is an acute phase protein produced from liver, showed higher levels in Chop−/− mice. Importantly, IL-1 and IL-6 are involved in regulating PAI-1 (31). This is consistent with increased Nlrp3 inflammasome activation and higher IL-1β level in Chop−/− mice.

Interestingly, the negative impact of Chop deletion on glucose homeostasis may depend on estrogen because female Chop^−/−^ mice with diet-induced obesity maintain glucose tolerance compared with WT controls, even though like male Chop^−/−^ mice, they have increased adiposity and hepatic steatosis (32). In response to pharmacologic ER stress, Chop activation decreases expression of CCAAT/enhancer binding protein α, peroxisome proliferator-activated receptor α, peroxisome proliferator-activated receptor γ, coactivator-1α, and sterol regulatory-element-binding protein-1 in hepatocytes (33). Although these factors both promote lipid synthesis and lipid degradation, global deletion of Chop appears to skew the balance toward liver lipid accumulation.

Surprisingly, Chop deletion during high fat feeding led to impaired glucose homeostasis, increased adipose tissue inflammation, and hepatic steatosis. The negative effects of Chop ablation may be mediated because an intact ER stress and apoptotic response is needed to adapt to a HFD. Loss of Chop may be beneficial when considering processes such as beta cell failure, but its potential contribution to adipose tissue and liver inflammation cannot be ignored. It also cannot be rule out that Chop may have cellular roles outside ER stress. Recently, X-box binding protein-1 has been shown to influence glucose homeostasis through targeting the degradation of forkhead box protein-1 to the 26 S proteasome, an effect independent of its role in ER stress signaling (34). There is interest in drugs targeting ER stress for management of glucose homeostasis. However, care must be taken in the pathways targeted because our data demonstrate that deletion of Chop signaling exacerbates adipose tissue inflammation and leukocytosis, resulting in systemic metabolic impairments.

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