Developmental Role of Macrophage Cannabinoid-1 Receptor Signaling in Type 2 Diabetes

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Islet inflammation promotes β-cell loss and type 2 diabetes (T2D), a process replicated in Zucker Diabetic Fatty (ZDF) rats in which β-cell loss has been linked to cannabinoid-1 receptor (CB1R)-induced proinflammatory signaling in macrophages infiltrating pancreatic islets. Here, we analyzed CB1R signaling in macrophages and its developmental role in T2D. ZDF rats with global deletion of CB1R are protected from β-cell loss, hyperglycemia, and nephropathy that are present in ZDF littermates. Adoptive transfer of CB1R-/- bone marrow to ZDF rats also prevents β-cell loss and hyperglycemia but not nephropathy. ZDF islets contain elevated levels of CB1R, interleukin-1β, tumor necrosis factor-α, the chemokine CCL2, and interferon regulatory factor-5 (IRF5), a marker of inflammatory macrophage polarization. In primary cultured rodent and human macrophages, CB1R activation increased Irf5 expression, whereas knockdown of Irf5 blunted CB1R-induced secretion of inflammatory cytokines without affecting CCL2 expression, which was p38MAPKα dependent. Macrophage-specific in vivo knockdown of Irf5 protected ZDF rats from β-cell loss and hyperglycemia. Thus, IRF5 is a crucial downstream mediator of diabetogenic CB1R signaling in macrophages and a potential therapeutic target.

Obesity is a risk factor for insulin resistance (IR), which can lead to progressive dysfunction and loss of pancreatic β-cells resulting in overt type 2 diabetes (T2D) (1,2), although β-cell dysfunction may arise independently, as it can precede the onset of IR (3). Adipose tissue inflammation plays a critical role in obesity-related IR (4), and a similar process associated with inflammatory cell infiltration in the endocrine pancreas has been linked to β-cell loss and the development of T2D (5).

Endocannabinoids (ECs) are endogenous ligands of cannabinoid receptors (cannabinoid-1 receptor [CB1R] and cannabinoid-2 receptor) that also mediate the effects of marijuana (6). The EC/CB1R system is overactive in obesity/metabolic syndrome (7,8), and blockade or genetic deletion of CB1R mitigates diet-induced obesity and its metabolic complications, including IR and T2D (reviewed by Mazier et al. [9]). CB1R blockade has similar beneficial effects in people with metabolic syndrome (10) or T2D (11) but can cause psychiatric side effects due to blocking CB1R in the central nervous system. ECs can inhibit hepatic insulin sensitivity via CB1R in the central nervous system (12) but can also inhibit insulin signaling directly via CB1R in adipose tissue (13), skeletal muscle (14), liver (15), and adipose tissue macrophages (16), and these latter targets account for the efficacy of peripherally restricted CB1R antagonists in mitigating IR (17,18).

Macrophage CB1Rs also play a prominent role in the progressive loss of β-cell function in Zucker Diabetic Fatty (ZDF) rats, a rodent model of T2D. The pancreatic islets of adult ZDF rats have reduced numbers of β-cells and are heavily infiltrated with proinflammatory macrophages expressing high levels of CB1R and the Nlrp3/Asc inflammasome (19).

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Peripheral CB$_1$R blockade, macrophage depletion, or macrophage-specific knockdown of CB$_1$R prevented these changes and preserved normoglycemia (19), which further illustrates the anti-inflammatory effect of CB$_1$R blockade. Chronic CB$_1$R blockade promotes a shift in the polarization of macrophages from proinflammatory to anti-inflammatory (19) and also reduces macrophage infiltration of diabetic islets by inhibiting the secretion of MCP-1 or CCL2 (19,20). ZDF rats also develop severe diabetic nephropathy associated with a loss of glomerular podocytes without significant macrophage infiltration or increase in Nlre3/ASC inflammasome expression in the kidney (21).

Together, these findings raise questions of whether there is an obligatory role for ECs in the development of T2D and its renal complication in the ZDF model. To this end, we have generated CB$_1$R-deficient rats on a ZDF background (ZDF-Cnr1 rats) and analyzed glycemic functions and renal parameters, as well as their modulation by adoptive bone marrow (BM) transfer. Our results indicate the obligatory requirement for peripheral CB$_1$Rs in both T2D and diabetic nephropathy, with CB$_1$R in BM-derived cells required for β-cell loss and the development of hyperglycemia, but not for podocyte loss and the resulting nephropathy.

Interferon regulatory factor-5 (IRF5) was recently implicated in polarizing macrophages toward the inflammatory phenotype (22), whereas mice with global or macrophage-specific deletion of If5 that were maintained on a high-fat diet remain insulin sensitive and display beneficial expansion of subcutaneous adipose tissue (23). Because of the unexpected similar expansion of subcutaneous but not visceral fat tissue observed in ZDF-Cnr1 rats, we explored the involvement of IRF5 in β-cell loss via CB$_1$R-mediated inflammatory signaling. Here we report that IRF5 mediates CB$_1$R-induced cytokine secretion and the resulting β-cell loss, whereas CB$_1$R-induced CCL2 production and macrophage transmigration is independent of IRF5 and involves activation of the α-isofrom of p38 mitogen-activated protein kinase (p38MAPK).

**RESEARCH DESIGN AND METHODS**

**Animals**

Animal protocols were approved by our institutional animal care and use committee. Male ZDF rats and their lean controls were obtained from Charles River Laboratories, housed individually under a 12-h light/dark cycle, and fed ad libitum a standard laboratory diet (STD; NIH-31 Rodent Diet).

**Generation and Characterization of ZDF-Cnr1 Rats**

A pair of zinc finger nucleases (Sigma-Aldrich, St. Louis, MO) was designed to cleave within the coding region of the Cnr1 gene, with the target site 5’-TACCACTTC ATCGCGAGCttggcaGTGGCCGACCTCCTG-3’ (zinc finger nuclease binding site set in capital letters). Identified founders carried an 11-base pair (BP) deletion located between T$^\text{17193}$ to C$^\text{17203}$ in the genomic DNA sequence.

**Genotyping**

Cnr1 and Fa genes were amplified as described in Supplementary Table 2.

**Drugs and Chemicals**

JD5037 was synthesized and its pharmacological properties analyzed as described previously (18). CP-55,940 was obtained from the NIDA Drug Supply Program (Research Triangle Park, NC). N-arachidonoylthanolamine (or anandamide [AEA]), arachidonoyl-2’-chloroethylamine (ACEA) and SP600125 were purchased from Cayman (Ann Arbor, MI). SB202190 was from Calbiochem (La Jolla, CA). All other chemicals were from Sigma-Aldrich.

**Serum and Urine Parameters**

Blood glucose levels were determined using the Elite Glucometer (Bayer, Pittsburgh, PA). Serum alanine aminotransferase (ALT), aspartate aminotransferase, free fatty acid (FFA), total cholesterol, triglycerides, insulin, proinsulin, C-peptide, glucagon, lepton, and adiponectin were quantified as described previously (19). IGF-I content was quantified with a Mouse/Rat IGF-I ELISA Kit (R&D Systems, Minneapolis, MN), whereas growth hormone was detected using a Rat/Mouse ELISA Kit (Millipore, Billerica, MA). Serum and urine creatinine, urea, and albumin concentrations and glomerular filtration rate were determined as described previously (21).

**Glucose Tolerance, Insulin Sensitivity Tests, HOMA-IR, and Glucose-Stimulated Insulin Secretion**

Glucose tolerance and insulin sensitivity tests were performed as described previous (24). HOMA-IR was calculated as follows: fasting insulin (µU/mL) × fasting glucose (mg/dL)/405 (25). Glucose-stimulated insulin secretion (GSIS) was determined as described previously (19).

**GTP$_\gamma$S Binding**

[^35$S$]GTP$_\gamma$S binding assay was performed as described (26).

**Whole-Body Irradiation and BM Transfer**

Whole-body irradiation was conducted as previously (27). Six-week-old male ZDF rats received 1 Gy total body irradiation from a $^{137}$Cs source. BM cells from ZDF and ZDF-Cnr1 donor rats were obtained as described previously (27). After a 2-h rest after irradiation, recipient rats were injected with $10^8$ BM cells via a tail vein. Animals were then rested for 2 weeks, and survivors with proper chimerism were used for experiments.

**Determination of Chimerism**

DNA was isolated from blood using a DNasey kit (69504; Qiagen, Germantown, MD). Cnr1 expression was detected using the genotyping protocol.

**Blood Cell Counts**

Blood samples were analyzed in an automated Hemavet blood analyzer (Drew Scientific Group, Miami Lakes, FL).

**Histology and Immunohistochemistry**

Pancreas, kidney, and adipose tissue were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned (4-µm sections) onto glass slides. Renal and adipose tissue histology were evaluated after hematoxylin-eosin and periodic acid Schiff staining. The antibodies used are listed in...
Supplementary Table 3. Slides were revealed by using the appropriate Elite ABC HRP (horse radish peroxidase)/diaminobenzidine (DAB) system (Vector Laboratories), counterstained with hematoxylin-eosin (Gills Formula; Vector Laboratories), or by secondary antibodies coupled to Alexa Fluor 488, 555, or 647. DAB slides were analyzed using an Olympus BX41 microscope, whereas fluorescent staining was analyzed using a Zeiss LSM700 confocal microscope. Immunopositivity was quantified using ImageJ software. Adipocyte diameters were evaluated digitally in light microscopy images of adipose tissue sections (n = 6–7 pictures per animal, three animals per group) using ImageJ software.

**TUNEL Staining**

TUNEL staining in pancreatic islet was performed using the Click-it TUNEL Alexa Fluor Imaging Assay from ThermoFisher Scientific (C10245).

**Isolation of Pancreatic Islets, β-Cell Mass**

Islets were isolated, and β-cell mass was determined as described previously (19).

**Islets Inflammation**

Caspase-1 activity was determined using the Caspase-1 Assay Kit (Fluorometric) from Abcam (ab39412); interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) proteins were measured using the Rat Quantikine ELISA Kit (R&D Systems).

**Cell Culture**

Human monocytic THP-1 cells were grown according to the recommendation of the American Type Culture Collection (ATCC). Cells were differentiated at 1.5–2 × 10^6 cells/well (35 mm) with 50 ng/mL phorbol myristic acid (P8139; Sigma-Aldrich) for 4 days. THP-1-derived macrophages were incubated in RPMI 1640 free of phorbol myristic acid, supplemented with 10% FBS (30–2020; ATCC), 50 µg/mL streptomycin, and 50 units/mL penicillin (30–2300; ATCC) for 24 h at 37°C, under an atmosphere of 5% CO2 in air prior to treatment. IL-1β, IL-18, IL-6, CCL2, and TNF-α protein concentrations were determined using ELISA kits from R&D Systems according to the manufacturer protocol.

**Small Interfering RNA Gene Knockdown**

Aliquots of peritonically elicited macrophages (PEO) or THP-1 cells were exposed for 48 h to siRNA listed in Supplementary Table 4. Control siRNAs were designed using the C911 technique (28).

**Preparation and In Vivo Administration of Glucan-Encapsulated siRNA Particles**

Fluorescein isothiocyanate–labeled glucan shells were prepared as described previously (29), and 8-week-old ZDF rats were then injected intraperitoneally every other day for 12 days with 6.17 mg/kg glucan-encapsulated siRNA particles (GeRPs) loaded with 308 nmol/kg endoporter and 0.410 mg/kg siRNA (Supplementary Table 4).

**Immunoblotting**

Cells were treated with pertussis toxin (PTX) 100 ng/mL, SB202190 25 µmol/L, SP600125 25 µmol/L, JD5037 100 nmol/L, and AEA and ACEA 1–10 µmol/L. Western blotting analyses were conducted as described previously (30), and antibodies used are listed in Supplementary Table 5.

**Real-Time PCR**

Total RNA extraction, reverse transcription, and real-time PCR were performed as described previously (19).

**Statistics**

If not otherwise specified, values are expressed as the mean ± SEM. Data were analyzed by Student t test or by one-way ANOVA followed by the Tukey-Kramer post hoc test. Time-dependent variables were analyzed, and results in multiple groups were compared by two-way ANOVA followed by Bonferroni test. Significance was set at P < 0.05.

**RESULTS**

**Generation and Characterization of Cnr1−/− Rats on a ZDF Background**

ZDF rats with a global knockout of CB1R (ZDF-Cnr1 rats) retained the mutation of the leptin receptor gene characteristic of Zucker fatty rats and ZDF rats and had an 11-BP deletion in the Cnr1 genomic DNA, as demonstrated by genotyping (Fig. 1A). Of note, the Cnr1 mutation led to a frameshift in the open reading frame resulting in a premature stop codon. As a result, CB1R protein was undetectable in the brain of ZDF-Cnr1 rats (Fig. 1B). In addition, the CB1R agonist CP-55940 increased [35S]GTPγS binding in plasma membranes prepared from ZDF but not from ZDF-Cnr1 brains (Fig. 1C), confirming the absence of CB1R signaling in the knockouts. ZDF-Cnr1 rats displayed lower food intake throughout the observation period and a slower rate of weight gain compared with their ZDF littermates but reached a similar body weight by the age of 26 weeks (Fig. 1D). Interestingly, the inguinal fat mass (often referred to as subcutaneous fat) was significantly larger in ZDF-Cnr1 than in ZDF rats, whereas the epididymal and perirenal fat pads, representing visceral fat, were of similar size (Fig. 1E). This was associated with a striking increase in the diameter of inguinal adipocytes in ZDF-Cnr1 versus ZDF rats, whereas an analogous difference in the size of perirenal adipocytes was much smaller (Supplementary Fig. 1).

The elevated serum levels of ALT, triglycerides, FFAs, and total cholesterol in ZDF rats were normalized in ZDF-Cnr1 rats (Fig. 1F). Additionally, the absence of Cnr1 resulted in normalization of the reduced plasma levels of growth hormone and IGF-I observed in ZDF rats (Fig. 1G). The hypoadiponectinemia of ZDF rats was reversed beyond the levels in lean controls, whereas the hyperleptinemia was unaffected by the deletion of Cnr1 (Fig. 1G).

**β-Cell Function Is Preserved and Hyperglycemia Prevented by Cnr1 Deletion**

ZDF rats develop extreme hyperglycemia due to β-cell loss by the age of 12–14 weeks (19). In contrast, ZDF-Cnr1 rats remained euglycemic for up to 26 weeks of age (Fig. 2A). At 8 weeks (i.e., before hyperglycemia starts to develop in ZDF
rats), ZDF-Cnr1 rats were more glucose tolerant than ZDF rats (Fig. 2B). In contrast, the two strains displayed equal IR in the insulin sensitivity test (Fig. 2C), as is also reflected by the similar degree of hyperinsulinemia and similar increases in HOMA-IR (Fig. 2D). Despite their similar IR, ZDF-Cnr1 rats have improved β-cell function, as reflected in the preserved GSIS, whereas ZDF rats were nonresponsive to a glucose challenge (Fig. 2E).

ZDF-Cnr1 rats exhibited normal plasma levels of glucagon in contrast to the hyperglucagonemia of ZDF
rats (Fig. 3A), which may contribute to the improved glucose tolerance in the presence of IR in ZDF-Cnr1 rats. Also, plasma proinsulin, insulin, and C-peptide levels were higher whereas the proinsulin/insulin ratio was significantly lower in ZDF-Cnr1 than in ZDF littermates (Fig. 3A), which is indicative of improved β-cell function and survival. The protection of β-cells in ZDF-Cnr1 rats was further confirmed by the higher islet insulin content (Fig. 3B). Of note, the strong infiltration of islets with CD68+ macrophages previously observed in younger, 14-week-old ZDF rats (19) was absent by the age of 26 weeks (Fig. 3B), probably due to the nearly complete destruction of islets by this age. Nevertheless, in a few remaining identifiable islets we could still observe strong infiltration of CD68+ macrophages (Fig. 3B, third row). In contrast, ZDF-Cnr1 rats maintained an almost normal islet structure without prominent CD68+ macrophage infiltration and had significantly larger β-cell mass than ZDF littermates (Fig. 3C). The higher β-cell mass inversely correlated with β-cell death, as reflected by the increased number of TUNEL-positive cells in ZDF compared with ZDF-Cnr1 islets (Supplementary Fig. 2A).

**ZDF-Cnr1 Rats Are Protected From Diabetic Nephropathy**

CB1R is a major effector in the development of diabetic nephropathy, and treatment of ZDF rats with a peripheral CB1R antagonist prevented or reversed this complication (21). Diabetic nephropathy, evident in 26-week-old ZDF rats by robust deterioration in renal parameters, was largely absent in ZDF-Cnr1 rats, as reflected in normalization of the albuminuria, glomerular filtration rate, and blood urea nitrogen (Supplementary Fig. 3A). Correspondingly, ZDF-Cnr1 rats were protected from podocyte loss, as demonstrated by Wilms tumor 1 immunostaining (Supplementary Fig. 3B). Furthermore, ZDF rats displayed glomerular enlargement and early mesangial matrix expansion that were absent in lean control or ZDF-Cnr1 rats. The pronounced albuminuria of ZDF rats was associated with prominent tubular protein resorption droplets and occasional tubular dilatation with proteinaceous casts. Again, no such changes were observed in ZDF-Cnr1 rats (Supplementary Fig. 3C).

**CB1R in Myeloid Cells Drive β-Cell Loss and Hyperglycemia but Not Nephropathy or Dyslipidemia**

Since macrophages are derived from BM myeloid precursors, we used the irradiation-BM transplantation (BMT) approach to test whether the lifelong β-cell protection in ZDF-Cnr1 rats is due to the absence of CB1R in macrophages. For total body radiation exposure from a 137Cs source, 1 Gy was the dose high enough to eliminate all circulating white blood cells without being lethal for at least 7 days after irradiation (Supplementary Fig. 4). Also, recipient rats needed at least 10⁸ donor BM cells to ensure survival. Animals were therefore irradiated at 6 weeks of age and transplanted 2 h after irradiation with 10⁸ BM cells pooled from three donor animals. Two weeks later, white blood cells were collected and DNA was extracted to verify the donor genotype. Five of seven recipients survived in each group, with all survivors achieving the expected chimerism (Fig. 4A).

BMT from ZDF donors to ZDF recipients tested for genotype-independent effects of irradiation and BMT. Recipients developed progressive hyperglycemia from 14 weeks on, reaching a plateau at 600 mg/dL by week...
The extent and rate of development of hyperglycemia were similar to those in control ZDF rats (dashed line), but its onset was delayed by 6 weeks, corresponding to the time it takes for the transferred cells to repopulate the irradiated BM and peripheral tissues (27). In contrast, ZDF rats receiving BM from ZDF-Cnr1 donors remained normoglycemic throughout the entire 20-week observation period and maintained higher plasma insulin and C-peptide levels compared with rats receiving ZDF BM (Fig. 4B). Interestingly, there was no difference in food intake or body weight between the two groups (Fig. 4B). CB1R protein was readily detectable postmortem in the BM of recipients of ZDF BM but was absent in recipients of ZDF-Cnr1 BM. As expected, CB1R was present at similar high levels in brain samples from both groups (Fig. 4C). Furthermore, CB1R highly colocalized with CD68+ macrophages in the islets of ZDF rats transplanted with ZDF BM, whereas there were fewer CD68+ cells and no detectable CB1Rs in islets of recipients of ZDF-Cnr1 BM (Fig. 4D). The absence of CB1Rs in hematopoietic cells was associated with higher pancreatic insulin content, normal pancreatic islet architecture, and reduced CD68+ macrophage infiltration, most likely due to reduced levels of the chemoattractant protein CCL2 (Fig. 4E). \( \beta \)-Cells in ZDF islets produce CCL2 (31,32) (Supplementary Fig. 2B), whereas the dramatically higher CCL2 expression in islets of ZDF rats transplanted with wild-type compared with Cnr1\(^{-/-}\) BM (Fig. 4E) suggests that transmigrating macrophages contribute to CCL2 secretion, as also supported by the ability of cultured macrophages to secrete CCL2 (see below) (Figs. 5 and 6).

In contrast, adoptive transfer of Cnr1\(^{-/-}\) BM did not influence the dyslipidemia (Supplementary Table 2) or the development of diabetic nephropathy (Supplementary Fig. 5A), including the loss of Wilms tumor 1-positive podocytes (Supplementary Fig. 5B).

**Distinct CB1R Signaling Pathways Involved in Chemokine and Inflammatory Cytokine Secretion by Macrophages**

Next, we analyzed the CB1R signaling pathways that mediate the diabetogenic functions of macrophages (19).
stimulation of rat PECs with the physiological CB1R agonist AEA (5 μmol/L) or its stable analog ACEA (5 μmol/L) activated p38MAPK and c-Jun N-terminal kinase (JNK) 2 without any detectable change in extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation (Fig. 5A). CB1R activation reduced STAT3 phosphorylation, a known corollary of M1 polarization (33), which was inversely related to p38MAPK activation (Fig. 5A). ACEA also induced the

Figure 4—CB1R ablation in myeloid cells protects ZDF rats from hyperglycemia. A: Time line for whole-body irradiation/BM transfer; genotyping by agarose gel chromatography of white blood cells (WBCs) from ZDF rats receiving ZDF BM (WTBM) or ZDF-KO BM (KOBM), with the 113-BP and 102-BP amplicons indicating Cnr1+/+ and Cnr1-/- genotype, respectively. B: Evolution of blood glucose and serum levels of insulin and C-peptide along with body weight and mean weekly food intake at 20 weeks in ZDF rats receiving wild-type BM (green) or Cnr1-/- BM (purple). Filled triangles and the dashed line indicates blood glucose levels in nonirradiated ZDF rats. C: Western blot of CB1R protein in brain and BM cells after killing of the rats at 20 weeks. D: Fluorescence immunohistochemistry of insulin, CB1-Rs, CD68, and colocalization analyzed by confocal microscopy. E: Increased insulin and decreased CD68 and CCL2 content of islets from ZDF rats receiving Cnr1-/- compared with wild-type ZDF BM. Columns and bars are the mean ± SEM from n = 5 animals/group. Scale bar, 100 μm. **P < 0.01, ***P < 0.001 relative to values in ZDF rats receiving wild-type BM (green columns).
secretion of the cytokines IL-1β and TNF-α and the chemokine CCL2, but failed to affect IL-6 production (Fig. 5B). SB202190, a potent inhibitor of p38MAPKα and p38MAPKβ, suppressed the secretion of CCL2 below control levels but did not affect TNF-α secretion, and a partial reduction of CB1R-evoked IL-1β secretion was not statistically significant (Fig. 5B). The JNK1/2/3 inhibitor SP600125, while fully abolishing c-Jun phosphorylation (data not shown), was only effective in inhibiting the ACEA-evoked CCL2 secretion (Fig. 5B). The effects of ACEA on PEC secretory responses were fully abolished by the CB1R inverse agonist JD5037 (Fig. 5B) and were similarly inhibited by siRNA-mediated selective knockdown of Cnr1 (Fig. 5C).

In the macrophage-differentiated human monocytic cell line THP-1, silencing CNR1 abolished ACEA-induced p38MAPK activation (Fig. 5D) and TNF-α, IL-1β, and CCL2 secretion (Fig. 5E). Knocking down MAPK14 (encoding p38MAPKα), but not MAPK11 (encoding p38MAPKβ),
completely blocked ACEA-induced secretion of CCL2 and significantly reduced the secretion of IL-1β but, again, failed to influence ACEA-induced TNF-α secretion (Fig. 5F and G). Thus, CB1R activation in macrophages engages p38MAPK to induce CCL2, but not TNF-α, secretion and partially contributes to IL-1β secretion.

Prompted by the similar selective expansion of subcutaneous, but not visceral, fat tissue in ZDF-Cnr1 rats (Fig. 1E) and in mice deficient in the proinflammatory transcription factor Irf5 (see Introduction), we explored the involvement of Irf5 in CB1R-mediated inflammatory signaling and β-cell loss. Irf5 gene expression was robustly increased in pancreatic islets isolated from ZDF compared with lean rats, whereas there was no such increase in islets of ZDF-Cnr1 rats or in islets of ZDF rats chronically treated with JD5037 (Fig. 6A). The involvement of CB1R was further confirmed in PECs isolated from wild-type mice, exposure of which to 1 μmol/L ACEA induced a threefold to fourfold increase in Irf5 mRNA, with no such effect in PECs from Cnr1−/− mice (Fig. 6B). Furthermore, in rat PECs, 1 μmol/L ACEA increased Irf5 mRNA twofold, an effect blocked by JD5037 or Ptx but not by SB202190 or SP600125 (Fig. 6C). Thus, CB1R regulates Irf5 expression via Gi/o-mediated inhibition of adenylate cyclase rather than activation of MAP kinases. In THP-1 macrophages, 1 μmol/L ACEA also caused an approximately twofold increase in Irf5 mRNA, and a similar effect was seen in THP-1 cells with siRNA-mediated knockdown of MAPK14, confirming the lack of involvement of p38MAPKα (Fig. 6D). SiRNA-mediated knockdown of Irf5 in rat PECs inhibited the ACEA-induced secretion of both TNF-α and IL-1β but had no effect on CCL2 secretion or Cnr1 expression (Fig. 6E), suggesting a requirement for Irf5 in CB1R-mediated cytokine production.

To explore the potential role of the CB1R/Gi/o/Irf5 signaling cascade in the loss of β-cell function in ZDF rats, 8-week-old ZDF rats were chronically treated with GeRP containing siRNA against rat Irf5, a technique that allows selective knockdown of target genes in phagocytic macrophages in vivo (19,34). We first optimized Irf5 siRNAs and selected one that yielded >90% knockdown of Irf5 gene expression in rat PECs after 48 h (Fig. 7A). ZDF rats were then treated intraperitoneally every other day

Figure 6—CB1R regulation of Irf5 expression and its role in β-cell loss in vivo. A: Irf5 mRNA levels in islets isolated from lean control (Ctrl) and ZDF rats treated for 4 weeks with vehicle (Veh) or 3 mg/kg/day JD5037. B: Irf5 mRNA levels in PECs isolated from wild-type or Cnr1−/− mice exposed to vehicle or 5 μmol/L ACEA for 24 h. C: Irf5 mRNA in rat PECs treated with vehicle or 5 μmol/L ACEA alone or in the presence of 100 nmol/L JD5037, 100 ng/mL Ptx, 25 μg/mL SB202190, or 25 μg/mL SP600125. D: ACEA-induced increase in Irf5 expression is not affected by MAPK14 knockdown. E: Effects of Irf5 knockdown on Cnr1 mRNA and on chemokine and cytokine secretion induced by 5 μmol/L ACEA in aliquots of 10⁶ rat peritoneal macrophages. Points/columns and bars are the mean ± SEM from six rats/group. Significant differences from values in Veh (*) or Veh + ACEA groups (#). *P < 0.05; **P < 0.01; ***P < 0.001; ##P < 0.01; ###P < 0.001.
for 12 days with GeRPs containing control siRNA or Irf5 siRNA. ZDF rats treated with control siRNA developed progressive hyperglycemia, whereas those receiving Irf5 siRNA maintained their pretreatment blood glucose level and also retained significantly higher plasma insulin and C-peptide levels (Fig. 7B). β-Cell protection was further indicated by the higher insulin content of islets from Irf5 siRNA-treated rats compared with control siRNA-treated rats (Fig. 7C). As expected, Irf5 knockdown significantly reduced Irf5 protein abundance in islets, whereas the number of CD68+ macrophages and CCL2 protein levels were similar in the two groups, indicating a lack of IRF5...
involvement in CCL2 secretion (Fig. 7C). Similarly, Irf5 knockdown did not affect Cnr1, CD68, and Cx32 mRNA levels in islets (Fig. 7D) but resulted in a >90% decrease in TNF-α content (Fig. 7E), accompanied by significant decreases in Pycard and Nlrp3 gene expression, caspase-1 activity, and IL-1β content (Fig. 7F). Furthermore, macrophage-specific in vivo knockdown of Irf5 resulted in an increased in CD3+ T lymphocytes (Supplementary Fig. 6A) associated with a decrease in T17 markers (Supplementary Fig. 6B) and an increase in T12 markers (Supplementary Fig. 6C), without a change in the T12 response (Supplementary Fig. 6D). Additionally, Irf5 knockdown did not affect Il12p40 or Il23 expression but did increase Il10 expression (Supplementary Fig. 6E).

DISCUSSION

Islet inflammation is a contributing factor to the progression of compensated IR into insulin-dependent T2D, and CB1R on proinflammatory macrophages play a prominent role in diabetic insulinis and loss of β-cell function in the ZDF model of T2D (19). To test the developmental role of CB1R in T2D, we generated CB1R-deficient rats on a ZDF background. Here we report that ZDF-Cnr1 rats are as hyperinsulinemic and insulin resistant as ZDF rats, but they are protected from the loss of β-cell function and consequently remain normoglycemic and are also protected from the associated nephropathy. Furthermore, adoptive transfer of ZDF-Cnr1 BM to ZDF recipients replicates the preservation of β-cell function and the associated nephropathy. This provides strong evidence that CB1Rs in BM-derived macrophages are both necessary and sufficient for the development of T2D but do not play a role in diabetic nephropathy in this model. However, we cannot exclude the possibility that glucotoxicity and lipotoxicity (35) also contribute to β-cell damage, either directly or by inducing the EC/CB1R system, as shown previously (19).

In contrast, the transfer of Cnr1−/− BM to ZDF rats did not mitigate diabetic nephropathy, indicating the involvement of a different cellular pool of CB1Rs. This is in agreement with the absence of significant infiltration of proinflammatory macrophages into glomeruli during the development of nephropathy in ZDF rats (21). A similar lack of involvement of proinflammatory macrophages has been indicated by the findings that selective deletion of Nlrp3 or caspase-1 expression in BM-derived cells failed to protect mice against the development of diabetic nephropathy, and the protective effect of the global deletion of Nlrp3 was not reversed by the transplantation of wild-type BM cells into Nlrp3-deficient mice (36).

The congruent effects of pharmacological blockade and genetic deletion of CB1Rs strongly support the diabeticogenic role of increased CB1R activity. This is different from the situation in leptin-deficient ob/ob mice, in which germline deletion of Cnr1 aggravated rather than mitigated their glucose intolerance (37). However, ob/ob mice on a C57BL/6J background compensate for IR by β-cell proliferation and thus do not become overtly diabetic, so the key pathogenic process promoted by CB1R activity in ZDF rats was absent in the murine model. On the other hand, the deletion of Cnr1 in both models failed to reverse basal hyperinsulinemia and IR, which is likely related to the leptin-deficient state. Leptin receptors are present on β-cells where they mediate increased KATP channel activity (38), and their activation was reported to decrease basal, but not glucose-stimulated, insulin secretion (39). Most importantly, we discovered that the transcription factor Irf5 is a key downstream mediator of CB1R-induced cytokine release by inflammatory macrophages and the resulting loss of β-cell function. First, Irf5 expression was robustly increased in the islets of ZDF compared with lean control rats. This increase was prevented by pharmacological blockade or genetic deletion of CB1R, which also prevented macrophage infiltration of islets,
suggesting that Irf5 expression was induced in macrophages. This was then further confirmed by the CB1R agonist–induced increase in Irf5 expression in primary cultured mouse and rat macrophages or in THP-1 cells. This effect was PTX sensitive but unaffected by p38MAPK or JNK blockade or knockdown, suggesting that it resulted from Gαo- and Gi-mediated inhibition of adenylate cyclase. Furthermore, siRNA-mediated knockdown of Irf5 in macrophages blunted the CB1R agonist–induced increase in TNF-α and IL-1β secretion and Nlrp3 expression, indicating the obligatory role of IRF5 in proinflammatory cytokine release. The anti-inflammatory response to Irf5 knockdown also involved a T helper 2-type immune response, as indicated by the increase in CD3+ T lymphocytes and the increased expression of the T helper 2 markers Gata3 and Il4 and decreased expression of the T helper 1 marker Tbet in pancreatic islets. These changes are similar to those found in adipose tissue of mice with diet-induced obesity (23) and are consistent with the proposed role of a T helper 1-type inflammatory response in T2D and its renal complications (40,41). Finally, macrophage-specific in vivo knockdown of Irf5 in ZDF rats protected β-cells and prevented the development of hyperglycemia, similar to the earlier reported effects of chronic CB1R blockade or selective knockdown of Cnr1 in macrophages (19).

IRF5, originally discovered as a transcription factor induced by type I interferons during viral infections (42), was more recently identified as a master regulator of macrophage M1 polarization and a mediator of obesity-related adipose tissue inflammation and the resulting IR (23). The present findings establish that the G-protein-coupled receptor CB1R is an upstream regulator of Irf5 expression and point to a broader metabolic function of macrophage IRF5 as a key driver of diabetogenic insulitis and β-cell loss.

Unlike CB1R blockade or gene deletion, which also reduced CCL2 chemokine secretion and the resulting transmigration of macrophages into islets (19), the knockdown of Irf5 failed to influence these parameters. This suggested that CB1Rs signal via an alternative, IRF5-independent pathway to promote CCL2 secretion. Indeed, CB1R agonists activated p38MAPK and JNK but not ERK1/2 in rodent and human macrophages, and inhibitors of p38MAPK or JNK blocked the parallel increase in CCL2 secretion without affecting the increased secretion of TNF-α or IL-1β. The siRNA-mediated knockdown of p38MAPKα, but not p38MAPKβ, in THP-1 macrophages similarly inhibited CB1R-induced CCL2, but not TNF-α secretion, and partially inhibited IL-1β secretion. This supports earlier findings that CB1R promotes CCL2 secretion via p38MAPK (43), but documents for the first time show the selective role of the p38MAPKα isoform in this effect.

Whereas the above findings indicate that inflammatory macrophages are an important source of CCL2, we could also detect this chemokine in β-cells, which is in agreement with evidence for CCL2 secretion by islet β-cells (31,32). It is possible that in the early stages of diabetic insulitis, β-cell–derived CCL2 initiates the transmigration of macrophages, which then amplify the production of this chemotactic signal. Another possible link between β-cells and macrophages is islet amyloid polypeptide, which is secreted by β-cells and stimulates inflammatory cytokine production by macrophages (44,45).

Together, the above findings demonstrate that activation of CB1Rs in proinflammatory M1 macrophages increases chemokine and cytokine secretion via distinct, partially overlapping signaling pathways, as illustrated in Fig. 8. The
CB2R-induced secretion of CCL2, but not TNF-α, is mediated via the activation of p38MAPKs. On the other hand, the CB2R-induced secretion of TNF-α, but not CCL2, is mediated via IRF5, and both pathways contribute to increased IL-1β secretion, with IRF5 having a more dominant role. The obligatory role of IRF5 in the secretion of cytotoxic cytokines thought to drive β-cell loss (19) is highlighted by the protective effect of macrophage-specific in vivo knockdown of Irf5. These findings mark IRF5 as a potential therapeutic target in T2D.

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Diabetes Volume 66, April 2017
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