Hematopoietic Tissue Factor—Protease-Activated Receptor 2 Signaling Promotes Hepatic Inflammation and Contributes to Pathways of Gluconeogenesis and Steatosis in Obese Mice

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Failure to inhibit hepatic gluconeogenesis is a major mechanism contributing to fasting hyperglycemia in type 2 diabetes and, along with steatosis, is the hallmark of hepatic insulin resistance. Obesity is associated with chronic inflammation in multiple tissues, and hepatic inflammation is mechanistically linked to both steatosis and hepatic insulin resistance. Here, we delineate a role for coagulation signaling via tissue factor (TF) and proteinase-activated receptor 2 (PAR2) in obesity-mediated hepatic inflammation, steatosis, and gluconeogenesis. In diet-induced obese mice, TF tail signaling independent of PAR2 drives CD11b⁺CD11c⁺ hepatic macrophage recruitment, and TF—PAR2 signaling contributes to the accumulation of hepatic CD8⁺ T cells. Transcripts of key pathways of gluconeogenesis, lipogenesis, and inflammatory cytokines were reduced in high-fat diet-fed mice that lack the cytoplasmic domain of TF (F3DCT) or that are deficient in PAR2 (F2rl1), as well as by pharmacological inhibition of TF—PAR2 signaling in diet-induced obese mice. These gluconeogenic, lipogenic, and inflammatory pathway transcripts were similarly reduced in response to genetic ablation or pharmacological inhibition of TF—PAR2 signaling in hematopoietic cells and were mechanistically associated with activation of AMP-activated protein kinase (AMPK). These findings indicate that hematopoietic TF—PAR2 signaling plays a pivotal role in the hepatic inflammatory responses, steatosis, and hepatic insulin resistance that lead to systemic insulin resistance and type 2 diabetes in obesity. (Am J Pathol 2015, 185: 524–535; http://dx.doi.org/10.1016/j.ajpath.2014.10.008)

The liver plays a central role in maintaining glucose homeostasis and lipid metabolism, and alterations in liver function underlie the development of cardiovascular and metabolic disease.1–3 A large proportion of obese patients develops hepatic steatosis, which leads to nonalcoholic fatty liver disease (NAFLD), a disease that ranges from fatty liver to the more severe condition of steatohepatitis, which can, if left unchecked, progress to fibrosis and cirrhosis in up to 25% of patients.4,5 Alterations of hepatic synthesis of procoagulant and prothrombotic pathway proteins observed in obese subjects with NAFLD further exacerbate the cardiovascular and metabolic risk in this population.6–9 Another feature of obesity and NAFLD is hepatic inflammation, reflected in the increased production of proinflammatory cytokines and acute-phase proteins and by the activation of pathways regulated by inhibitor of nuclear factor kappa-B kinase subunit beta (IKK-β) and Jun N-terminal kinase (JNK).10,11 Recent studies suggest a role for hepatic inflammation and immune cells in the regulation of both NAFLD and hepatic insulin resistance. A macrophage population distinct from resident Kupffer cells is recruited to the liver during obesity, and proinflammatory activation of liver macrophages is linked to both insulin resistance and NAFLD.12,13 However, mechanisms that contribute to the inflammatory state of the obese liver and pathways that drive the recruitment of

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macrophages and other immune cells, including T cells, remain poorly characterized.

Strong clinical evidence suggests that a TF-mediated coagulation pathway is up-regulated in obesity and in metabolic syndrome. Obese subjects have higher plasma concentrations of coagulation factor VII (FVII), increased levels of thrombin and thrombin—antithrombin complexes, and increased circulating monocye TF procoagulant activity.\(^{14-16}\) TF initiates the coagulation cascade by serving as the cell surface receptor for FVIIa. In addition to its coagulation functions, TF activates signaling cascades with numerous roles in both normal physiology and disease.\(^{17-19}\) The TF–FVIIa complex initiates direct TF signaling by activating proteinase-activated receptor 2 (PAR2) and regulates cell migration via a mechanism involving phosphorylation of the TF cytoplasmic domain.\(^{20}\) Pharmacological and genetic approaches using an antibody (10H10) that specifically blocks TF–PAR2 signaling or mice that lack the cytoplasmic domain of TF or PAR2-deficient mice have provided evidence for TF–PAR2 signaling in cancer progression,\(^{19,21}\) angiogenesis,\(^{21,22}\) and inflammation.\(^{23-25}\) Using such pharmacological and genetic approaches we have established a role for TF–PAR2 signaling in the development of obesity and insulin resistance.\(^{26}\) We have shown that nonhematopoietic cell TF–VIIa–PAR2 signaling promoted obesity via regulation of overall metabolism and energy expenditure.

By contrast, TF–PAR2 signaling in hematopoietic cells attenuated adipose tissue macrophage recruitment and inflammation and led to increased systemic insulin sensitivity. Although these studies suggested that TF-mediated adipose dysfunction may contribute to obesity and systemic insulin resistance, the contribution of hepatic TF signaling to systemic glucose homeostasis and hepatic dysfunction (NAFLD and hepatic insulin resistance) remained to be determined. In the present study, we used genetic and pharmacological tools to unravel a role for TF and PAR2 signaling in hepatic steatosis and insulin resistance. Here, we show that hematopoietic TF–PAR2 signaling blunts AMPK activation, increases hepatic inflammation, and activates pathways of lipogenesis and gluconeogenesis that contribute to hepatic steatosis and insulin resistance, respectively. At the cellular level, TF and PAR2 regulate hepatic recruitment and proinflammatory activation of a population of CD11b\(^{+}\)CD11c\(^{-}\) macrophages and CD8\(^{+}\) T cells. Our findings suggest that limiting steatosis and hepatic glucose production by inhibiting TF–PAR2 signaling may provide a novel therapeutic approach for the treatment of type 2 diabetes driven by obesity.

**Materials and Methods**

**Mice**

Mice lacking the TF cytoplasmic domain (TF\(^{ΔCT}\)), PAR2-deficient mice (Flt1\(^{-/-}\); hereafter Par2\(^{-/-}\)), and TF\(^{ΔCT}/Par2\(^{-/-}\) mice were all in the C57BL/6J background. Humanized TF knock-in mice (TFKI) in the C57BL/6J background were from Dr. Mark Anderson (Johnson & Johnson Pharmaceutical Research & Development, Spring House, PA). Male mice were fed either a high-fat diet (HFD) (60% kcal from fat) or a low-fat diet (LFD) (10% kcal from fat) (Research Diets, New Brunswick, NJ) beginning at 6 to 8 weeks of age. Bone marrow (BM) chimeras were generated by injecting 5 \(\times\) 10\(^6\) to 10 \(\times\) 10\(^6\) BM cells at 4 to 6 hours after lethal irradiation of mice. After engraftment under antibiotic prophylaxis for 6 weeks, mice were fed a HFD for 16 to 20 weeks. BM donors typically carried a transgene for green fluorescent protein (GFP) [C57BL/6-Tg(CAG-EGFP)131Osb/LeySopJ; Jackson Laboratory, Bar Harbor, ME], and reconstitution efficiency was determined by analyzing GFP positivity in isolated liver mononuclear cells.

All experiments were approved by the animal ethics committee of the Torrey Pines Institute for Molecular Studies and the Institutional Animal Care and Use Committee of the Scripps Research Institute.

**Plasma Insulin, Glucose, and Insulin Tolerance Test**

Fasting plasma insulin levels were measured using a mouse ultrasensitive insulin enzyme-linked immunosorbent assay kit (catalog number 80-INSMSU-E01; ALPCO Diagnostics, Salem, NH) according to the manufacturer’s instructions. Plasma glucose was measured with a glucometer (Bayer, Leverkusen, Germany). To test for insulin tolerance, mice were injected with insulin (0.75 U/kg; Eli Lilly, Indianapolis, IN), and glucose concentrations were measured in blood samples from tail bleeds at baseline and at 15, 30, 60, 90, and 120 minutes after injection.

**Western Blot Analysis**

Total protein was extracted from snap-frozen livers in the presence of protease and phosphatase inhibitors, and equal amounts of proteins were separated by 10% SDS PAGE and transferred to nitrocellulose membranes. The membranes were probed with antibodies to AMPK\(^{\alpha}\) and phosphorylated AMPK\(^{\alpha}\) (p-AMPK\(^{\alpha}\) T172) (Cell Signaling Technology, Danvers, MA). Immunoreactive proteins were visualized using an Amersham enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, UK). For analysis of insulin-dependent Akt phosphorylation, mice were injected with 0.75 U/kg insulin via the tail vein 10 minutes before harvesting liver samples for Western blotting using antibodies to Akt and p-Akt (Cell Signaling Technology).

**Quantification of Tissue and Plasma Triglyceride**

Liver samples (20 to 30 mg) were homogenized for 10 minutes in 1 mL isopropanol using a Bio-Gen Pro200
homogenizer (Pro Scientific, Oxford, CT) and centrifuged at 2000 \(\times g\) for 10 minutes. From the recovered supernatant, 100 \(\mu L\) was dried in a SpeedVac vacuum system (Thermo Fisher Scientific, Waltham, MA); the resulting pellet was dissolved in isopropanol and used for determination of triglyceride content with a triglyceride E test (Wako Chemicals USA, Richmond, VA). Plasma triglyceride was measured directly using the Wako triglyceride E test according to the manufacturer’s instructions.

### Isolation of Liver MNCs, Flow Cytometry, and Cell Selection

Mouse livers were harvested and finely diced with a new scalpel in phosphate-buffered saline and homogenized in a six-well plate using a plunger; the resulting cell suspension was passed through a 100-µm nylon cell strainer (BD Falcon; BD Biosciences, San Jose, CA). Cells were washed with phosphate-buffered saline and pelleted via centrifugation at 400 \(\times g\) for 10 minutes at 4°C. The supernatant was discarded, and cells were suspended in 36% Percoll (GE Healthcare). This suspension was thoroughly mixed and centrifuged at 500 \(\times g\) for 10 minutes at room temperature with the brake off. The supernatant was removed gently, and the resulting pellet was washed with red blood cell lysis buffer before repelleting and collection of liver mononuclear cells (MNCs).

For flow cytometry, MNCs were blocked with anti-FcR (eBioscience, San Diego, CA) and stained with fluorescence-labeled monoclonal antibodies (eBioscience) in the dark at 4°C for 30 minutes. Cells were analyzed on a Becton Dickinson FACSCalibur (BD Biosciences) using FlowJo software version 10 (Tree Star, Ashland, OR). For cell fractionation, liver MNCs were incubated with CD8 or CD11b microbeads (Miltenyi Biotec, Auburn, CA), and corresponding CD8+ or CD11b+ cells were enriched by positive magnetic-activated cell sorting according to the manufacturer’s protocol (Miltenyi Biotec).

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**Figure 1**  Hematopoietic TF--PAR2 signaling contributes to gluconeogenesis and insulin resistance during diet-induced obesity. A and B: TF (A) and PAR2 (B) mRNA in the liver of male C57BL/6J WT mice fed a LFD (10% fat) or a HFD (60% fat) for 16 weeks. C and D: Hepatic G6Pase (C) and PEPCK-1 (D) mRNA in LFD-fed WT mice and HFD-fed WT, TF<sup>−/−</sup>, Par2<sup>−/−</sup>, and double-deficient TF<sup>ACT</sup>/Par2<sup>−/−</sup> mice. E: Western blot analysis of insulin-induced p-Akt levels in liver from HFD-fed WT, TF<sup>ACT</sup>, or Par2<sup>−/−</sup> mice. F: HFD-fed TFKI mice (16 weeks) were treated intraperitoneally with either 20 mg/kg anti-human TF signaling-selective monoclonal antibody 10H10 or IgG control twice a week for 3 weeks while continuing on the HFD, after which fasting plasma glucose and insulin were measured and the mice were subjected to an insulin tolerance test. For this test, mice were injected with 0.75 U/kg insulin, and blood glucose was determined at intervals from 0 to 120 minutes. Relative expression of liver G6Pase and PEPCK-1 mRNA was also tested. G: G6Pase and PEPCK-1 mRNA in liver from HFD-fed (16 weeks) WT, TF<sup>ACT</sup>, or Par2<sup>−/−</sup> BM chimeras in C57BL/6J WT mice. H: Hepatic G6Pase and PEPCK-1 mRNA expression from DIO TFKI BM chimeras at 24 hours after receiving 20 mg/kg anti-human TF 10H10, compared with obese TFKI BM chimeras receiving nonspecific IgG. Data are expressed as means ± SD. n = 8 to 12 (A–D); n = 5 (E); n = 8 to 10 (G); n = 6 to 8 (H). *P < 0.05, **P < 0.01, and ***P < 0.001. BM, bone marrow; DIO, diet-induced obesity; DKO, double-deficient (TF<sup>ACT</sup>/Par2<sup>−/−</sup>); G6P, G6Pase; HFD, high-fat diet; LFD, low-fat diet; PAR2, proteinase-activated receptor 2; TF, tissue factor; TF<sup>ACT</sup>, lacking the cytoplasmic domain of TF; TFKI, TF knock-in; WT, wild type.
Hematopoietic TF—PAR2 signaling increases pathways that drive steatosis during diet-induced obesity. A and B: Hepatic SREBP-1c and FAS mRNA in LFD-fed WT and HFD-fed WT, TF<sup>ΔCT</sup>, Par2<sup>-/-</sup>, and TF<sup>ΔCT</sup>/Par2<sup>-/-</sup> double-deficient mice. C: HFD-fed TFKI mice (16 weeks) were treated intraperitoneally with either 20 mg/kg anti-human TF signaling selective monoclonal antibody 10H10 or IgG control twice a week for 3 weeks while continuing on HFD; hepatic SREBP-1c and FAS mRNA levels were then determined. D: Hematoxylin and eosin-stained section of liver, with liver and plasma triglyceride levels from HFD-fed TFKI mice treated with either IgG or 10H10 (as in C). E: SREBP-1c and FAS mRNA in liver from 16 weeks HFD-fed WT, TF<sup>ΔCT</sup>, or Par2<sup>-/-</sup> BM chimeras in C57BL/6J WT mice. F: Hepatic SREBP-1c and FAS mRNA expression from DIO TFKI BM chimeras at 24 hours after receiving 20 mg/kg anti-human TF 10H10, compared with obese TFKI BM chimeras receiving nonspecific IgG. Data are expressed as means ± SD. n = 8 to 12 (A and B); n = 8 (C and D); n = 8 to 10 (E); n = 6 to 8 (F). *P < 0.05, **P < 0.01, and ***P < 0.001. DKO, double-deficient (TF<sup>ΔCT</sup>/Par2<sup>-/-</sup>).

RT-qPCR
cDNAs synthesized from total RNA (TRIzol reagent; Life Technologies, Carlsbad, CA) were analyzed by real-time quantitative PCR with reverse transcription (RT-qPCR) with gene-specific primer sets (Life Technologies) and SYBR Green PCR master mix (PerkinElmer, Waltham, MA) in an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA).<sup>26</sup> Relative gene expression levels were calculated after normalization to 18S rRNA using the ΔΔC<sub>T</sub> method (Bio-Rad Laboratories). The 18S expression was stable in the samples measured. Separate control experiments demonstrated that the efficiencies of target and reference (ie, 18S) amplifications were equal, thus validating the use of ΔΔC<sub>T</sub> for all of the transcripts measured.

Statistical Analysis
Statistical significance of differences between two groups was analyzed using the unpaired Student’s t-test.

Results
Hematopoietic TF—PAR2 Signaling Contributes to Pathways that Drive Gluconeogenesis and Steatosis during Diet-Induced Obesity

Although under normal conditions insulin suppresses hepatic glucose production (gluconeogenesis), the ability of insulin to shut down hepatic glucose production is diminished during a state of hepatic insulin resistance, resulting in hyperglycemia and type 2 diabetes. To determine the contribution of TF and PAR2 signaling to hepatic insulin resistance during obesity, we initially determined whether these proteins are induced in the liver during weight gain. Compared with wild-type (WT) mice fed a LFD for 16 weeks, WT mice fed a HFD had higher levels of TF and PAR2 mRNA in the liver (Figure 1, A and B). A major mechanism by which insulin inhibits hepatic glucose output is regulation of the expression of rate-limiting gluconeogenic genes, such as PCK1 [encoding phosphoenolpyruvate
higher in HFD-fed WT mice (Figure 1, C and D). Importantly compared with WT HFD-fed WT mice, mice deficient in the cytoplasmic domain of TF (TF<sup>KI</sup>) mice exhibited improvements in systemic insulin sensitivity as indicated by reduced levels of fasting plasma glucose, and improved sensitivity in the insulin tolerance test (Figure 1F). Similarly, in our previous studies, we had shown that insulin sensitivity was improved in HFD-fed TF<sup>ΔCT</sup> or Par2<sup>−/−</sup> mice, as well as in HFD-fed BM chimeras deficient in either TF cytoplasmic domain or PAR2 in hematopoietic cells. The improvements of...

paradoxically blocks TF-β PAR2 signaling but not the downstream TF-mediated coagulation. TF knock-in (TFKI) mice carrying human TF under the control of the endogenous mouse TF promoter were fed a HFD for 8 weeks. Weight gains in these mice were similar to those of WT mice (data not shown). Groups of DIO TFKI mice were then treated with 10H10 (20 mg/kg) or vehicle twice weekly for 3 weeks while continuing on the HFD. Compared with vehicle-treated TFKI control mice, the 10H10-treated TFKI mice exhibited improvements in systemic insulin sensitivity, as indicated by reduced levels of fasting plasma insulin, glucose, and improved sensitivity in the insulin tolerance test (Figure 1F).

To directly link TF—PAR2 signaling to regulation of gluconeogenesis in the liver, we used a monoclonal antibody (10H10) to human TF that specifically blocks TF—VIIa—PAR2 signaling but not the downstream TF-mediated coagulation. TF knock-in (TFKI) mice carrying human TF under the control of the endogenous mouse TF promoter were fed a HFD for 16 weeks. C and D: FACS analysis and quantification of CD8<sup>−</sup> and CD4<sup>+</sup> T cells from liver of LFD-fed and HFD-fed WT mice. E: TF mRNA expression in CD8<sup>+</sup> selected and CD11b<sup>+</sup> selected cells from liver of HFD-fed (16 weeks) DIO C57BL/6J WT mice. F: Quantification of TF expression in CD11b<sup>−</sup>CD11c<sup>+</sup> versus CD11b<sup>+</sup>CD11c<sup>−</sup> macrophage populations in liver of HFD-fed DIO WT mice. G: PAR2 mRNA expression in CD8<sup>−</sup> versus CD11b<sup>+</sup> selected cells from liver of HFD-fed DIO WT mice. H: FACS analysis of GFP-labeled CD11b<sup>−</sup>CD11c<sup>+</sup> and CD11b<sup>+</sup>CD11c<sup>−</sup> macrophage populations from liver of HFD-fed WT receiving BM from GFP mice. Data are expressed as means ± SD. n = 4 (A–H). *P < 0.05; **P < 0.01. FACS, fluorescence-activated cell sorting.

carboxykinase 1 (PEPCK-1) and G6PC [encoding glucose-6-phosphatase (G6Pase)]. Compared with WT LFD-fed mice, transcripts of hepatic G6Pase and PEPCK-1 were higher in HFD-fed WT mice (Figure 1, C and D). Importantly, compared with WT HFD-fed WT mice, mice deficient in the cytoplasmic domain of TF (TF<sup>ΔCT</sup>), PAR2, or both had lower levels of both G6Pase and PEPCK-1 (Figure 1, C and D). The contribution of TF and PAR2 to hepatic insulin resistance was further analyzed by measuring insulin-mediated phosphorylation of Akt in the liver of HFD-fed TF<sup>ΔCT</sup> or Par2<sup>−/−</sup> mice. Compared with HFD-fed WT mice, both TF<sup>ΔCT</sup> and Par2<sup>−/−</sup> mice had significantly higher expression levels of liver p-Akt in response to acute insulin injection (Figure 1E).

To directly link TF—PAR2 signaling to regulation of gluconeogenesis in the liver, we used a monoclonal antibody (10H10) to human TF that specifically blocks TF—VIIa—PAR2 signaling but not the downstream TF-mediated coagulation. TF knock-in (TFKI) mice carrying human TF under the control of the endogenous mouse TF promoter were fed a HFD for 8 weeks. Weight gains in these mice were similar to those of WT mice (data not shown). Groups of DIO TFKI mice were then treated with 10H10 (20 mg/kg) or vehicle twice weekly for 3 weeks while continuing on the HFD. Compared with vehicle-treated TFKI control mice, the 10H10-treated TFKI mice exhibited improvements in systemic insulin sensitivity, as indicated by reduced levels of fasting plasma insulin, glucose, and improved sensitivity in the insulin tolerance test (Figure 1F). Similarly, in our previous studies, we had shown that insulin sensitivity was improved in HFD-fed TF<sup>ΔCT</sup> or Par2<sup>−/−</sup> mice, as well as in HFD-fed BM chimeras deficient in either TF cytoplasmic domain or PAR2 in hematopoietic cells. The improvements of...
systemic insulin sensitivity observed in long-term (3 weeks) 10H10-treated TFKI mice were also associated with reduced levels of hepatic transcription of G6Pase and PEPCK-1 (Figure 1F). Thus, TF
$^e$
PAR2 signaling drives pathways that contribute to increased glucose output and hepatic insulin resistance during DIO.

BM chimeras were used to determine whether hematopoietic TF—PAR2 signaling contributes to gluconeogenesis during obesity. HFD-fed mice deficient in the TF cytoplasmic domain or PAR2 in hematopoietic cells also showed reduction in hepatic G6Pase and PEPCK-1 (Figure 1G). The contribution of hematopoietic TF—PAR2 signaling to gluconeogenesis was further analyzed using TFKI mice to generate BM chimeras in which hematopoietic cells were humanized for TF. In these chimeric mice, human TF is expressed only in hematopoietic cells, thus allowing us to inhibit TF—VIIa—PAR2 signaling specifically in this cellular compartment with the anti-human TF antibody 10H10 or IgG control twice a week for 3 weeks. Data are expressed as means ± SD, $n = 4$ (A–D); $n = 8$ (E and F). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

In a previous study, we found that hepatic steatosis was significantly reduced in HFD-fed TF$^{ΔCT}$ or Par2$^{−−/−}$ mice, and this phenotype was recapitulated in HFD-fed BM chimeras deficient in either TF cytoplasmic domain or PAR2 in hematopoietic cells. However, we had not determined the mechanisms by which TF and PAR2 signaling contributes to steatosis. In the present study, gene expression of the lipogenic transcription factor sterol regulatory element-binding protein 1c (SREBP1c) and its target enzyme, fatty acid synthase (FAS), was increased in HFD-fed WT mice but was significantly reduced in HFD-fed TF$^{ΔCT}$, Par2$^{−−/−}$, and double-deficient TF$^{ΔCT}$/Par2$^{−−/−}$ mice (Figure 2, A and B). Reductions were also observed for these lipogenic mediators in the livers of DIO TFKI mice in response to long-term treatment with 10H10 (Figure 2C), which provided direct evidence for TF—PAR2 signaling in this response. Direct examination of steatosis

Figure 4  TF and PAR2 contribute to hepatic immune cell recruitment in obesity. A–D: FACS analysis and quantification of liver CD11b$^+$CD11c$^+$ and CD11b$^+$CD11c$^+$ macrophage populations (A and B) and CD8$^+$ and CD4$^+$ T-cell populations (C and D) from male WT, TF$^{ΔCT}$, or Par2$^{−−/−}$ mice fed a HFD for 16 weeks. E and F: FACS analysis and quantification of liver macrophages (E) and T cells (F) from DIO TFKI mice treated with 20 mg/kg anti-human TF antibody 10H10 or IgG control twice a week for 3 weeks. Data are expressed as means ± SD. $n = 4$ (A–D); $n = 8$ (E and F). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.
by histological examination and biochemical analysis revealed significantly reduced levels of triglyceride accumulation and/or steatosis in 10H10-treated DIO TFKI mice; however, plasma triglyceride levels remained unchanged in 10H10-treated mice (Figure 2D). These findings suggest that one of the mechanisms by which TF−PAR2 signaling contributes to steatosis is up-regulation of de novo lipogenesis and also that TF−PAR2 does not appear to contribute hepatic triglyceride secretion.

The phenotype with reduced SREBP1c and FAS was recapitulated in TF and PAR2 hematopoietic signaling-deficient HFD-fed BM chimeric mice and in HFD-fed TFKI BM chimeras acutely treated with the antibody 10H10 (Figure 2, E and F). Thus, hematopoietic TF−PAR2 signaling drives lipogenic pathways that contribute to hepatic steatosis in obesity.

TF and PAR2 Contribute to Hepatic Immune Cell Recruitment in Obesity

Although a number of studies suggest that hepatic inflammation contributes to hepatic insulin resistance and NAFLD, mechanisms and cell-specific contributions of hepatic inflammation to the pathogenesis of these liver disorders are unclear. Recent reports have also described a myeloid population that is recruited to the liver from circulating monocytes during the development of obesity. Nonetheless, the distinct functions of recruited monocyte-derived hepatic macrophages and other immune cells to obesity-associated hepatic dysfunction are unclear.

To determine whether TF and PAR2 signaling contributes to hepatic immune cell recruitment, we initially characterized the changes in the repertoire of macrophages and T cells in the liver of WT mice in response to a HFD. FACS analysis revealed an increase in the percentage of macrophages positive for the surface markers CD11b and CD11c and a decrease in the percentage of CD11b+CD11c− cells in the liver of HFD-fed WT mice, compared with mice fed a LFD (Figure 3, A and B). The percentage of CD8+ T cells was further increased, but that of CD4+ T cells was lower, in the liver of DIO HFD WT mice (Figure 3, C and D), relative to lean LFD WT mice. We next determined the relative expression of TF and PAR2 in the CD11b+ and CD8+ cell populations in livers from DIO WT mice. FACS analysis indicated that the CD11b+ cell populations was predominantly (70%) composed of macrophages, and that CD8+ cells were predominantly (90%) T cells (Supplemental Figure S1). CD11b+ and CD8+ cell populations were isolated using paramagnetic microbeads coated with antibodies to CD11b and CD8, respectively. Fractionation efficiency indicated efficient purification of these cell populations (Supplemental Figure S2), with minimal crossover. TF expression was higher in the hepatic CD11b+ population (predominantly macrophages), relative to the CD8+ population (mostly T cells) (Figure 3E). FACS analysis indicated that, in macrophages, TF was higher in the CD11b+CD11c− subpopulation relative to CD11b+CD11c+ cells (Figure 3F). By contrast, PAR2 gene expression was relatively higher in the CD8+ T cells than in the CD11b+ macrophages (Figure 3G).

To determine whether these macrophage populations were recruited from hematopoietic cells from BM, we generated BM chimeric mice in which BM was transplanted from WT GFP donor mice to WT non-GFP recipients. The chimeric mice were fed a HFD for 16 weeks and then immune cell populations recruited to the liver were determined by
FACS analysis. Both the CD11b+CD11c+ and the CD11b+CD11c− populations in the liver were predominantly GFP+ (Figure 3H), which indicates that these macrophage populations represent hematopoietic populations recruited to the liver during DIO and are distinct from resident Kupffer cells, which are long-lived and radiation-resistant self-renewing embryo-derived local macrophages. 27

Compared with HFD-fed WT mice, the percentage of CD11b−CD11c+ macrophages was decreased in HFD-fed TFΔCT mice, but not in HFD-fed Par2−/− mice (Figure 4, A and B). No changes were observed in the relative levels of CD11b+CD11c− cells in HFD-fed TFΔCT or Par2−/− mice (Figure 4, A and B), compared with WT, but CD8+ T cells decreased and CD4+ T cells increased (Figure 4, C and D). To directly determine the immune cells that are recruited to the obese liver via TF–PAR2 signaling, we treated DIO TFKI mice for 3 weeks with 10H10 to block TF–PAR2 signaling. The various immune populations were determined using FACS analysis. In these TFKI mice, a specific reduction was observed only for the CD8+ T-cell population (Figure 4, E and F). Taken together, these results suggest that TF tail signaling independent of PAR2 drives CD11b+CD11c+ hepatic macrophage recruitment and that TF–PAR2 signaling contributes to CD8+ T cell recruitment.

Figure 6 Hematopoietic TF–PAR2 signaling contributes to hepatic inflammation in obesity. mRNA expression of TNF-α, IFN-γ, and IL-10 from HFD-fed (16 weeks) WT, TFΔCT, or Par2−/− BM chimeras in C57BL/6J WT mice (A–C) or in liver of DIO TFKI BM chimeras 24 hours after receiving 20 mg/kg anti-human TF 10H10, compared with obese TFKI BM chimeras receiving nonspecific IgG (D–F). Data are expressed as means ± SD. Data are expressed as means ± SD. n = 8 to 10 (A–C); n = 6 to 8 (D–F). *P < 0.05, ***P < 0.001.

Figure 7 TF–PAR2 signaling increases inflammatory responses in hepatic immune cells. A–C: mRNA expression of TNF-α, IFN-γ, and IL-10 in CD8+ selected and CD11b+ selected cells from liver of HFD-fed WT C57BL/6J mice. D–F: mRNA expression of TNF-α and IFN-γ in CD8+ selected cells (D and E) and mRNA expression of IL-10 in CD11b+ selected cells (F) from liver of HFD-fed WT, TFΔCT, or Par2−/− mice. Data are expressed as means ± SD. n = 3. *P < 0.05, **P < 0.01, ***P < 0.001.
TF and PAR2 Contribute to Proinflammatory Responses of Hepatic Immune Cells

To establish the contribution of TF and PAR2 signaling in hepatic inflammation, we initially determined the transcription of key inflammatory genes in liver from mice fed either a LFD or a HFD for 16 weeks. Expression of TNF-α, IFN-γ, and IL-10 was increased in total liver RNA extracts from WT HFD-fed mice, compared with their lean LFD-fed counterparts (Figure 5, A–C). To confirm the contribution of TF–PAR2 signaling to this inflammatory profile, we treated DIO TFKI mice long term with 10H10. In these mice, TNF-α and IFN-γ gene expression was reduced, and IL-10 expression was increased (Figure 5, D–F), indicating that TF–PAR2 signaling contributes to the hepatic proinflammatory changes that accompany obesity.

Because our BM transplantation studies indicated that TF and PAR2 signaling in hematopoietic cells drives pathways of hepatic insulin resistance and steatosis (Figures 1 and 2), we analyzed whether these changes in inflammatory gene expression are mediated by hematopoietic TF–PAR2 signaling. HFD-fed BM chimeras deficient in the TF cytoplasmic domain or PAR2 in hematopoietic cells exhibited significantly lower levels of hepatic TNF-α and IFN-γ mRNA, but increased expression of IL-10 (Figure 6, A–C). HFD-fed TFKI BM chimeric mice with human TF in the hematopoietic compartment were treated acutely with 10H10 to specifically inhibit TF–PAR2 signaling in this compartment. Measures of hepatic inflammatory gene expression in these mice also indicated decreased levels of TNF-α and IFN-γ mRNA, but IL-10 levels were induced (Figure 6, D–F). Taken together, these results suggest that TF–PAR2 signaling in hematopoietic cells contributes to hepatic inflammation during DIO obesity.

To determine the contribution of TF and PAR2 signaling in macrophages and CD8+ T cells in the inflammatory response during DIO, we measured hepatic inflammatory gene expression in these immune cells in DIO WT mice. Notably, expression of TNF-α and IFN-γ was higher in CD8+ cells (relative to the CD11b+ macrophage population) in the livers of DIO WT mice (Figure 7, A and B), but expression of these cytokines in CD8+ cells was significantly lower in TFΔCT and Par2−/− mice (Figure 7, D and E). By contrast, IL-10 gene expression was relatively higher in CD11b+ cells, compared with CD8+ cells (Figure 7C). IL-10 expression in CD11b+ cells was further increased in TFΔCT and Par2−/− mice (Figure 7F).

TF–PAR2 Signaling Blunts Hepatic AMPK Activation

AMPK plays crucial roles in the regulation of hepatic glucose and lipid metabolism and is a major target for the treatment of metabolic disease.28–30 Enhancing hepatic AMPK activity reduces hepatic insulin resistance and steatosis via ameliorating pathways of gluconeogenesis and inhibiting lipogenesis, respectively.31–36 However, regulatory mechanisms of AMPK in the liver are not well understood. Because PAR2 signaling is known to blunt AMPK activity via the β-arrestin pathway,37 we hypothesized that, in obesity, TF–PAR2 signaling in the liver may blunt AMPK activity and thereby lead to the downstream effects of hepatic insulin resistance and steatosis. To test this hypothesis, we measured AMPK activity (phosphorylation at threonine 172) in liver of TFKI mice that were treated for 3 weeks with 10H10 to inhibit TF–PAR2 signaling. Western blot analysis indicated that hepatic AMPK activity was significantly increased in 10H10-treated TFKI mice, compared with TFKI mice treated with IgG (Figure 8A). To determine whether hematopoietic TF–PAR2 signaling similarly blunts AMPK activity, we treated DIO TFKI BM chimeras acutely with 10H10 and analyzed hepatic AMPK activity. p-AMPK was

![Figure 8](http://ajp.amjpathol.org)
significantly increased in the liver of DIO TFKI BM chimeric mice treated with 10H10, compared with control IgG-treated mice (Figure 8B).

Discussion

Obesity, a major risk factor for type 2 diabetes, is often linked with abnormal regulation of hepatic glucose production and steatosis. The liver is crucial for maintaining glucose homeostasis and is the major site of endogenous glucose production. Inadequate insulin-mediated suppression of hepatic gluconeogenesis is a manifestation of hepatic insulin resistance and is pivotal in the pathogenesis of type 2 diabetes. We had demonstrated that mice lacking TF cytoplasmic domain or PAR2 in the whole body or only in the hematopoietic compartment showed improved glucose tolerance, insulin sensitivity, and decreased hepatic steatosis.26 Although these improvements were associated with reductions in adipose in glucose homeostasis and steatosis remained unclear. With signaling in the liver to improvements in whole-body chimeric mice treated with 10H10, compared with control.

Deletion of PPAR—

Diabetes. We had demonstrated that mice lacking TF cytoplasmic domain or PAR2 in the whole body or only in the hematopoietic compartment showed improved glucose tolerance, insulin sensitivity, and decreased hepatic steatosis.26 Although these improvements were associated with reductions in adipose in glucose homeostasis and steatosis remained unclear. With the present study, we have demonstrated that hepatic TF—PAR2 signaling promotes pathways of lipogenesis and gluconeogenesis that contribute to steatosis and hepatic insulin resistance, respectively.

Transcripts of lipogenic (SREBP1c, FAS) and gluconeogenic (PEPCK-1, G6Pase) proteins were lower in the livers of HFD-fed mice lacking the TF cytoplasmic domain or PAR2 in hematopoietic cells and in response to inhibition of hematopoietic TF—PAR2 signaling in DIO mice, indicating a crucial role for TF—PAR2 signaling in the hepatic metabolic abnormalities associated with obesity. Previous studies have shown that coagulation signaling mediated by downstream thrombin—PAR1 contributed to TNF-α and MCP-1 expression, but not steatosis, in mice fed a diet deficient in methionine and choline.38 In mice fed a Western diet, PAR1 and hematopoietic-derived TF contributed to hepatic inflammation and steatosis.8 Similar to adipose inflammation, hepatic inflammation involving inflammatory responses in local immune cells is linked to pathways that drive hepatic insulin resistance and NAFLD in response to obesity.12,13 However, neither the inflammatory response in the obese liver with respect to macrophages and other immune cells nor the signals that activate their proinflammatory responses are well characterized. In addition to Kupffer cells (resident macrophages in liver), obesity promotes the recruitment of a morphologically and functionally distinct monocyte-derived macrophage cell population.13 Depletion of both Kupffer cells and recruited hepatic macrophages using gadolinium or clodronate shows that liver macrophages are a causal factor in obesity-induced hepatic insulin resistance.39 Deletion of PPARδ within hematopoietic cells polarizes the liver resident macrophages (Kupffer cells) toward an M1 classically activated state and reduces systemic insulin sensitivity.12 In these studies, however, the specific contributions of Kupffer cells versus recruited macrophages to the observed phenotype is unclear. Gadolinium and clodronate deplete all macrophages, but hematopoietic deletion of PPARδ is expected to delete PPARδ only in the population of recruited macrophages, and not in resident Kupffer cells, which are long-lived and radiation-resistant self-renewing embryo-derived cells.27

In the present study, a population of macrophages positive for the surface markers CD11b and CD11c was significantly increased in the liver in response to a HFD. BM transplantation studies with GFP-labeled donors suggest that positivity for CD11b and CD11c defines the population that is recruited to the liver in response to a HFD, a population distinct from resident Kupffer cells. Mice deficient in the TF cytoplasmic domain (TFΔCT) had decreased levels of CD11b⁺CD11c⁻ macrophages, but no changes in this macrophage population was observed in Par2−/− mice. Thus, TF cytoplasmic tail signaling contributes to hepatic macrophage recruitment independent of PAR2. The mechanism of TF-mediated macrophage recruitment to the obese liver is consistent with other systems in which TF mediates integrin-dependent signaling and cell migration independent of PAR2.18,20,40,41 FACS analysis indicated that a HFD also led to a significant increase in hepatic CD8⁺ T cells, which may also drive local inflammation. In this instance, both TFΔCT mice and Par2−/− mice exhibited decreased levels of hepatic CD8⁺ T cells in response to obesity. Inhibition of TF—PAR2 signaling in DIO TFKI mice similarly reduced CD8⁺ T cells, which suggests a direct role for TF—PAR2 signaling in the recruitment of hepatic CD8⁺ T cells. In DIO WT mice, levels of proinflammatory cytokines TNF-α and IFN-γ were higher in hepatic CD8⁺ cells (predominantly CD8⁺ T cells), relative to CD11b⁺ cells (primarily macrophages). CD8⁺ cell expression of these cytokines was reduced in TFΔCT and Par2−/− mice. By contrast, levels of the anti-inflammatory cytokine IL-10 were higher in hepatic CD11b⁺ cells, relative to CD8⁺ cells, in DIO WT mice. IL-10 levels in CD11b⁺ cells were further increased in HFD-fed TFΔCT mice and Par2−/− mice.

Because of the morphological location of Kupffer cells within the liver, the mechanical procedure used to homogenize the liver for fractionation of CD8⁺ and CD11b⁺ cell populations appears to predominantly isolate the recruited BM-derived immune cells. FACS analysis indicated that the majority (approximately 90%) of all liver MNCs isolated from GFP⁺ BM transplanted donors are GFP⁺ (data not shown). Thus, this procedure does not appear to result in a significant isolation of resident Kupffer cells, which are long lived and radiation resistant. These results suggest a model in which TF tail signaling contributes to hepatic macrophage recruitment and TF—PAR2 signaling regulates macrophage IL-10 expression. By contrast, TF—PAR2 signaling contributes to hepatic CD8⁺ T cell accumulation, as well as TNF-α—mediated and IFN-γ—mediated inflammatory pathways, in this cell population.
Our present findings suggest that hematopoietic AMPK may link TF–PAR2 signaling to hepatic inflammation and downstream pathways of gluconeogenesis and lipogenesis. In addition to its role in regulation of gluconeogenesis and lipogenesis,11−36,42−44 AMPK has potent anti-inflammatory effects.45 It suppresses NF-kB signaling,26 and hematopoietic AMPK regulates obesity-induced inflammation and whole-body glucose homeostasis.47 AMPK is the major target for the widely prescribed anti-diabetic drug metformin, which inhibits hepatic gluconeogenesis, reduces blood glucose, and improves lipid and whole-body metabolism.48,49 Despite such metabolically favorable actions of AMPK, its regulatory mechanisms are not fully understood. In addition to its role in regulation of gluconeogenesis and lipogenesis,31,36,42−44 TF signaling via inhibition of PAR2 may provide promising therapies to reverse chronic inflammation, glucose and lipid abnormalities associated with obesity, and type 2 diabetes.

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Supplemental Data
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References


