Profilin-1 Haploinsufficiency Protects Against Obesity-Associated Glucose Intolerance and Preserves Adipose Tissue Immune Homeostasis

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Metabolic inflammation may contribute to the pathogenesis of obesity and its comorbidities, including type 2 diabetes and cardiovascular disease. Previously, we showed that the actin-binding protein profilin-1 (pfn) plays a role in atherogenesis because pfn heterozygote mice (PfnHet) exhibited a significant reduction in atherosclerotic lesion burden and vascular inflammation. In the current study, we tested whether pfn haploinsufficiency would also limit diet-induced adipose tissue inflammation and insulin resistance (IR). First, we found that a high-fat diet (HFD) upregulated pfn expression in epididymal and subcutaneous white adipose tissue (WAT) but not in the liver or muscle of C57BL/6 mice compared with normal chow. Pfn expression in adipose tissue correlated with F4/80, an established marker for mature macrophages. Of note, HFD elevated pfn protein levels in both WAT and adipocytes of C5/BL/6 mice compared with normal chow. Pfn expression in adipose tissue correlated with F4/80, an established marker for mature macrophages, which were also preferentially biased toward an M2-like phenotype; this correlated with preserved frequency of regulatory T cells. Taken together, the findings indicate that pfn haploinsufficiency protects against diet-induced IR and inflammation by modulating WAT immune homeostasis. Diabetes 62:3718–3726, 2013

Chronic low-grade inflammation accompanies and may contribute to the pathogenesis of obesity and its comorbidities, including type 2 diabetes (T2D) (1,2) and cardiovascular disease (3). Although its molecular bases remain incompletely understood, metabolic inflammation can be found at both the systemic and the tissue level and is characterized by activation of immune cells and abnormal production of cytokines and chemokines (4). Infiltration of macrophages and other immune cells has been invoked as a pathogenetic factor in white adipose tissue (WAT) inflammation and expansion, resulting in systemic insulin resistance (IR) (5). Although operationally grouped in M1 and M2 polarization extremes, adipose tissue macrophages (ATMs) are highly plastic and heterogeneous, resulting in overlapping phenotypes of activation (6). CD11c⁺ ATMs are highly enriched in obesity, particularly in the crown-like structures (CLSSs) that surround dead or dying adipocytes. It has also been proposed that CD11c⁺ ATMs express high levels of proinflammatory mediators and promote IR (7,8). ATMs expressing the galactose-type C-type lectin (MGLL/CD301), albeit in part overlapping with CD11c⁺ ATMs (9), are enriched in the interstitium of lean and obese WAT and have been suggested to display additional M2-like features (9,10).

Studies have begun to clarify the networks of interactions between ATMs and adipocytes (11) and other infiltrating immune cells within obese WAT. Specifically, WAT expansion is associated with an increased ratio of CD8⁺ to CD4⁺ T cells, which precedes ATM accumulation (12–14). Additionally, decreased frequency of Foxp3⁺ regulatory T cells (Treg) was noted in the WAT of both ob/ob mice and mice fed a high-fat diet (HFD) (13,14). Depletion of Treg promoted IR and upregulation of inflammatory cytokines in WAT, whereas stimulation of Treg function using interleukin (IL)-2–based complexes partially protected against HFD-induced IR (15). In addition, reconstitution with CD4⁺ cells but not CD8⁺ improved the metabolic phenotype of Rag1-null mice, which are deficient in B and T lymphocytes and display accelerated IR (14). Together, these studies suggest that Treg maintain immune homeostasis within the WAT microenvironment.

Diet-induced accumulation of macrophages and other immune cells in WAT offers conspicuous analogies with the activation of both innate and adaptive immunity in the vascular wall during atherogenesis (15). We described an essential role for the actin-binding protein profilin-1 (pfn) in early atherosclerotic lesion formation (16). Pfn is essential for early development as pfn homozygous knockout die at the two-cell stage (17); it is best characterized by its critical function in actin dynamics (18,19) and regulation of adhesion and migration in numerous cell types, including macrophages (20). Postdevelopmental expression of pfn is highest in macrophages and adipocytes, intermediate in hepatocytes and endothelial cells (ECs), and nearly undetectable in striate muscle (16, G.R.R., unpublished observations). Although it resides primarily intracellularly and lacks a signal peptide, pfn can be secreted extracellularly through exosomes (21), which are 60- to 90-nm vesicles originated by late endosomes; can be detected in the serum of patients with atherosclerosis (22); and can activate the innate immune response to parasites in dendritic cells (23). Several lines of evidence suggest that pfn levels are increased in diabetes and vascular inflammation. The diabetic milieu, which is associated with a proinflammatory/reactive state (24), can upregulate pfn expression in mesangial cells (25). Additionally, we showed...
that pfn levels are increased in the aorta of diabetic rats (26,27), in human atherosclerotic lesions (22), and in cultured ECs exposed to oxidized cholesterol (oxChol) through activation of the transcription factor signal transducer and activator of transcription-3 (STAT3) (27).

In the current study, we addressed whether in addition to a role in atherogenesis pfn plays a role in diet-induced WAT inflammation and IR. We found that pfn heterozygote mice (PfnHet) are protected against HFD-induced systemic IR, ATM accumulation, and WAT inflammation. Additionally, HFD-fed PfnHet display preserved frequency of Treg in association with ATMs biased toward an M2 anti-inflammatory phenotype.

**RESEARCH DESIGN AND METHODS**

C57BL/6J mice were purchased from Taconic and fed an HFD (60% calories from fat) (Harlan Teklad) starting at 7 weeks of age or maintained on normal chow (NC) (10% calories from fat) (Harlan Teklad) for the indicated intervals before testing or euthanasia. In other experiments, mice were obtained after having been maintained on HFD or NC at the Taconic facility.

PfnHet and wild-type littermates (PfnWT) were originally provided by Walter Witke (Mouse Biology Program, European Molecular Biology Laboratory, Monterotondo, Italy) on a C57BL/6J background (Pfn homozygous knockout die at the two-cell stage) (17). Both mouse strains were further backcrossed on a C57BL/6J background for at least five generations before being used for experiments. Genotyping was performed as described elsewhere (16). Beginning at 7 weeks of age, PfnHet and PfnWT were fed an HFD or continued on NC for the indicated intervals. At the time of euthanasia, tissues and blood were collected for analysis. All mice had free access to water and chow and were housed under alternating 12-h light and dark cycles. All experiments were approved by the Joslin Diabetes Center Animal Care and Use Committee and were performed in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals and U.S. Department of Agriculture regulations under the Animal Welfare Act.

**Metabolic parameters and circulating metabolites.** Blood was collected from tail veins of unanesthetized mice after an overnight fast to measure glucose (Glucometer Elite; Bayer) and serum insulin (Crystal Chem, Downers Grove, IL). One hundred microliters of serum was obtained from overnight-fasted mice to assay cytokines and chemokines (Quansys Bioscience, Logan, UT) with a Luminex ELSA multiplex platform. For glucose tolerance testing (GTT), PfnWT and PfnHet males fed HFD or NC for 12 weeks were injected intraperitoneally with 2.0 g glucose/kg body weight, and blood glucose level was measured at indicated times.

**Isolation of adipocytes and stromal vascular fraction.** Epididymal adipose tissue was processed for isolation of adipocytes and stromal vascular fraction (SVF) as described previously (29). Briefly, epididymal fat pads were excised and minced into Krebs-Ringer bicarbonate (KRB) solution (12.5 mmol/L HEPES [pH 7.4], 120 mmol/L NaCl, 6 mol/L KCl, 1.2 mmol/L MgSO4, 1 mol/L CaCl2, 2% BSA, and 2.5 mmol/L glucose) followed by digestion with collagenase type II 1 mg/mL (Worthington, Lakewood, NJ) and DNase I 0.2 mg/mL (Sigma, St. Louis, MO) at 37°C for 20 min with shaking. The solution containing digested adipose tissue was filtered through a 250-μm nylon sieve to remove large particles and centrifuged at 300 g for 5 min to separate floating adipocytes from the SVF pellet. Floating adipocytes were washed again with KRB solution containing 5 mmol/L ethylenediaminetetraacetic acid (EDTA) and centrifuged again at 300 g for 5 min to separate residual SVF, which was pooled with the initial SVF. Adipocytes were washed again with KRB solution, centrifuged, and collected for protein or RNA extraction. SVF pellets were treated with ammonium-chloride-potassium lysing buffer (Invitrogen, Grand Island, NY) to remove red blood cells.

**Flow cytometry.** SVF pellets were resuspended in 2% FBS/PBS containing 1% Fe Block (BD Pharmingen, San Diego, CA) and stained with cocktails of conjugated antibodies (Supplementary Table 1). After staining and washing with 2% FBS/PBS, cells were filtered through a 100-μm mesh and stained with propidium iodide (Sigma) at a final concentration of 0.2 μg/mL to exclude dead cells. ATMs were defined as F4/80+CD11b+ cells within the CD45+, CD3–, B220–, TER119–, and NK1.1– population (Supplementary Fig. 1), and in selected experiments, they were sorted by Aria (BD Biosciences) for quantitative real-time PCR (qRT-PCR) or Western blotting. For ATM subset analysis, cells were further stained with monoclonal antibodies specific for CD11c, MHC-II, Ly6C (eBioscience, San Diego, CA), CD206, and CD301/MGL-1 (AbD Serotec, Raleigh, NC). For intracellular staining of Foxp3, the Foxp3 staining buffer set (eBioscience) was used according to the manufacturer’s protocol. Briefly, cells were incubated with Fe Block for 15 min and then stained for 20 min with a cocktail of antibodies (Supplementary Table 1). Labeled cells were

![FIG. 1. Pfn expression in WAT. A: qRT-PCR shows a significant increase for pfn in epididymal (Epi) and subcutaneous (Subc) WAT from 19-week-old C57BL/6 mice fed HFD for 12 weeks compared with NC (n = 8). **P < 0.0001; *P < 0.005. B: Pfn and F4/80 expression positively correlated in Epi WAT of HFD for 12 weeks (R² = 0.703, P < 0.0001). C: qRT-PCR shows a significant increase for pfn in Epi fat from 12-week-old C57BL/6 mice fed HFD for 6 weeks compared with NC (n = 8/group). *P < 0.05. D: Pfn expression in Epi fat correlated with F4/80 both in mice fed NC (R² = 0.52, P < 0.05) and in mice fed HFD (R² = 0.91, P < 0.0001) for 6 weeks.](diabetes.diabetesjournals.org)
washed with 2% FBS/PBS, fixed and permeabilized with 1% formaldehyde (Polysciences, Inc.) for 10 min at room temperature followed by an addition of 750 μL 1 mol/L glycine (final concentration 0.15 mol/L) for 5 min at room temperature to stop fixation. The tissue was washed in PBS and 2% FBS and minced with scissors. SVF was isolated as described previously. Epididymal fat pads pooled from three HFD- or eight NC-fed mice were used for each chromatin immunoprecipitation (ChIP), yielding approximately 6 million stromal vascular cells (SVCs) per pool. The final SVF pellet was resuspended in 350 μL SDS lysis buffer (50 mmol/L Tris-Cl [pH 8.0], 10 mmol/L EDTA, 1% SDS), and incubated for 15 min on ice. Protease inhibitors leupeptin (10 μmol/L), pepstatin (1 μmol/L), aprotinin (5 μg/mL), and phenylmethylsulfonyl fluoride (1 mmol/L) (all from Sigma) were added to the buffers throughout the procedure. Sonication was then performed on ice with a Branson Sonifier 450 (five cycles of 10-s pulses at a constant setting, one-fourth of maximum power, interspersed by 30-s pauses) to yield DNA fragments of ~0.3–0.7 kbp in size. After sonication, DNA fragments were purified by QiAquick spin columns (Qiagen, Valencia, CA). SYBR green custom primers were used to amplify three fragments of 5′-untranslated region (UTR) of the mouse pfn-1 gene (Supplementary Table 3), including a 406 region encompassing the STAT response element TT(CCCCG)AA located at −513 to −505. Quantification of the immunoprecipitated chromatin and input was carried out as described elsewhere in this section. Level of target chromatin was normalized by input.

**FIG. 2.** ChIP in SVF ex vivo. Whole epididymal fat pads were fixed with formaldehyde immediately after dissection. After SVF isolation, ChIP was performed with the use of anti-STAT3, CEBP-β, and anti-acetylated histone H3 (AcH3) (kbp 9–14) antibodies followed by gel electrophoresis of 50% of the associated DNA (A) and qRT-PCR of the remainder (B). Negative control is chromatin incubated with nonimmune rabbit IgG. Input indicates the level of specific target chromatin before immunoprecipitation and was used to normalize qRT-PCR values. A: Representative gel electrophoresis showing that HFD induces STAT3 recruitment to a ~300-bp fragment of pfn 5′-UTR (~711 to ~406), encompassing a single STAT3 response element (~505 to ~513) in association with increased lysine acetylation of H3 (arrow). B: The quantification of the chromatin bound to candidate transcription factors within the pfn promoter. Compared with NC, HFD promotes a statistically significant STAT3 recruitment to the fragment of pfn 5′-UTR (~711 to ~406) that encompasses a single STAT3 response element (~505 to ~513) as well as increased lysine acetylation of H3 of the same fragment, suggesting transcriptional activation. CEBP-β did not associate with a more proximal fragment of pfn 5′-UTR encompassing two response elements (~302 to ~315 and ~182 to ~192, respectively). For each ChIP, chromatin was pooled from three HFD or eight NC mice (n = 3–5 pools/group). *P < 0.0001. IP, immunoprecipitate.

**FIG. 3.** Effects of HFD on pfn protein levels. A: Sections from epididymal WAT of NC- and HFD-fed mice were stained with anti-pfn antibody followed by colorimetric detection (brown). Staining was detected in SVCs of HFD samples (arrows) and was more intense in cells surrounding adipocytes. Slides were counterstained with hematoxylin (inset, blue). The negative control is the HFD section stained with nonimmune IgG. Scale bar: 50 μm. Western blot analysis of SVC (B) and adipocyte (C) extracts from epididymal fat pads of 18-week-old mice fed HFD for 12 weeks. HFD induced a three- to fourfold pfn increase in both compartments. Pfn densitometric values were normalized to tubulin. +, RAW264.7 macrophage (n = 6/group, P < 0.0001 for both SVCs and adipocytes at 12 weeks, 2.7-fold increase in both compartments). Pfn densitometric values were normalized to tubulin. +, RAW264.7 macrophage (n = 6/group, P < 0.0001 for both SVCs and adipocytes at 12 weeks, 2.7-fold increase in both compartments). Pfn densitometric values were normalized to tubulin. +, RAW264.7 macrophage (n = 6/group, P < 0.0001 for both SVCs and adipocytes at 12 weeks, 2.7-fold increase in both compartments). Pfn densitometric values were normalized to tubulin. +, RAW264.7 macrophage (n = 6/group, P < 0.0001 for both SVCs and adipocytes at 12 weeks, 2.7-fold increase in both compartments). Pfn densitometric values were normalized to tubulin. +, RAW264.7 macrophage (n = 6/group, P < 0.0001 for both SVCs and adipocytes at 12 weeks, 2.7-fold increase in both compartments). Pfn densitometric values were normalized to tubulin. +, RAW264.7 macrophage (n = 6/group, P < 0.0001 for both SVCs and adipocytes at 12 weeks, 2.7-fold increase in both compartments).
Immunohistochemistry and morphometric analysis. Immunohistochemistry and morphometric analysis was performed by 5-μm-thick formalin-fixed, paraffin-embedded epididymal adipose sections, which were cut at the Histology Core from the Joslin Diabetes Center. Staining with anti-F4/80 antibody 150 (Serotec) was performed overnight at 4°C followed by a peroxidase-based Vectastain ABC Kit (Vector Laboratories, Burlingame, CA), as described previously (29,30). CLS density was expressed as the number of CLSs per 400 adipocytes (31) counted from three sections cut at least 50 μm apart. Consecutive sections were stained, with nonimmune rat IgG2b as negative control. Sections were counterstained with hematoxylin before morphometric analysis under light microscopy by an independent observer in a masked fashion.

Adipocyte area was calculated from the same three sections used to count CLSs as 50 random adipocytes per section (total of 150 adipocytes per mouse). Adipocytes within CLSs were not included in this analysis. Images were collected and analyzed with a Zeiss Axioskop2 Mot Plus microscope equipped with Axiovision software.

Western blot. Proteins from mouse tissues and sorted macrophages were extracted in radioimmunoprecipitation assay buffer and processed for immunoblot analysis as described elsewhere (26).

RNA extraction and qRT-PCR. RNA was isolated from sorted ATMs with QIAzol (Qiagen) and amplified with a MessageAMP II kit (Ambion, Austin, TX). For whole adipose tissue, liver, and soleus muscle, RNA was isolated with an RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer’s instructions. One microgram of total RNA was converted to double-stranded cDNA with an Advantage RT for PCR kit (Clontech, Palo Alto, CA). Primers used in RT-PCR were from Applied Biosystems (Foster City, CA) and are listed in Supplementary Table 4. cDNA levels were measured with TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI 7900HT RT-PCR system (Applied Biosystems). Levels of target genes were normalized by TATA binding protein (TBP).

Statistical analysis. Data are presented as mean ± SD. Unpaired t test was used for comparisons between two groups, whereas ANOVA followed by Bonferroni test was used for comparison for multiple groups.

RESULTS

WAT pfn expression is increased by HFD and correlates with macrophage accumulation. The attenuated accumulation of macrophages in the vascular wall and the atheroprotective phenotype exhibited by PfnHet prompted us to test whether pfn also played a role in diet-induced IR, ATM accumulation, and WAT inflammation. Because we had previously detected increased levels of pfn in the aorta of mice fed a high-cholesterol diet (16) and in ECs exposed to oxChol (27), we first investigated the effects of HFD on pfn expression in other metabolically relevant candidate tissues in the current study. Compared with NC, feeding with HFD for 12 weeks resulted in a statistically significant elevation of pfn mRNA in epididymal and, to a lesser extent, subcutaneous WAT but not in liver or muscle (Fig. 1A). In epididymal WAT, mRNA expression of pfn closely correlated with that of F4/80, an established marker for mature macrophages (32). The upregulation of pfn in epididymal WAT (Fig. 1C) and the correlation between pfn and F4/80 expression (Fig. 1D) were already noted after 6 weeks of HFD, suggesting that these are early events in the natural history of WAT inflammation rather than the consequence of obesity-associated WAT remodeling. Of note, the correlation between pfn and F4/80 expression was somewhat weaker but also statistically significant in mice fed NC (Fig. 1D).

HFD induces STAT3 recruited to the pfn promoter in SVF ex vivo. To address the transcriptional mechanisms for HFD-induced pfn gene expression, we optimized an ex vivo strategy for ChIP in SVF. In rat ECs, ChIP of the pfn 5'-UTR showed that STAT3 recruitment to a single response element at −501 was indispensable for oxChol-mediated pfn upregulation (27). In SVF isolated from the epididymal WAT of mice fed NC or HFD for 12 weeks, STAT3 was recruited to the mouse pfn promoter in an HFD-dependent manner. Additionally, activation of the same region of pfn, estimated by histone 3 lysine acetylation, was markedly enhanced by HFD (Fig. 2A and B). In
parallel experiments, binding of other candidate transcription factors with response elements within the 5'-UTR (up to \( \sim 1,110 \)), such as c-Rel (data not shown) or CEBP-\( \beta \), was not detected (Fig. 2B). Thus, similar to the effects of oxChol in cultured ECs (27), HFD elicited the recruitment of STAT3 to the \( \text{pfn} \) promoter in SVCs.

**HFD elevates \( \text{pfn} \) protein levels in both the adipocyte and the SVF compartment.** Next, we investigated distribution of the \( \text{pfn} \) protein level in WAT compartments. Staining of 5-\( \mu \)m sections of WAT with a specific anti-\( \text{pfn} \) antibody showed adipocytes from both NC and HFD-fed mice and revealed a strong immunoreactivity of SVC in HFD-fed mice (Fig. 3A). Of note, \( \text{pfn} \) staining within the SVC population was more intense in cells surrounding degenerating adipocytes (Fig. 3A, *inset*), suggesting that upregulation of \( \text{pfn} \) was predominant in typical CLS. Additionally, \( \text{pfn} \) levels were measured in protein extracts obtained from the SVF and the buoyant adipocyte fraction of epididymal WAT. Compared with NC, feeding with HFD for 12 weeks elicited a three- to fourfold elevation of \( \text{pfn} \) in both SVF and adipocyte fraction (Fig. 3B and C). This increase was already detected to a lesser extent after 8 weeks of HFD (Fig. 3C). Taken together, these results indicate that HFD induces a significant elevation of \( \text{pfn} \) protein levels in both SVCs and adipocytes and that \( \text{pfn} \) expression significantly correlates with ATM accumulation in the early phases of obesity.

**\( \text{Pfn} \) haploinsufficiency protects against diet-induced IR.** These experiments set the foundation to test the effect of \( \text{pfn} \) dosage on diet-induced IR and inflammation. Seven-
week-old PfnWT and PfnHet were maintained on NC or HFD for varying time periods. As mentioned previously, pfn homozygotes could not be used because they die during embryonic development. As expected, HFD elicited a significant elevation in triglyceride and free fatty acid levels that was comparable in PfnWT and PfnHet (data not shown). Body weight was similar in the two groups under NC and HFD conditions (Supplementary Fig. 3). Of note, PfnHet were largely protected from the HFD-induced elevation of fasting insulin (FI) levels observed in PfnWT (12-week HFD 1.01 ± 0.16 vs. 2.1 ± 0.46 ng/mL) (Fig. 4A), whereas fasting blood glucose (FBG) levels were similar between the two groups and remained in the normal range until 12 weeks of HFD. Together, elevated FI and normal FBG levels suggested that HFD induced a state of compensated IR. Homeostasis model assessment for IR (HOMA-IR), an index of insulin sensitivity (33), mirrored the pro-

**PfnHet show blunted ATM accumulation and formation of CLS but unchanged adipocyte function.** We reasoned that the protection against HFD-induced IR in PfnHet could be, at least in part, a result of taming inflammation in WAT and systemically. Because the expression of pfn correlates with that of F4/80 in epididymal WAT (Fig. 1B and D), we first tested whether ATM accumulation would be reduced in PfnHet. Indeed, flow cytometry analyses of SVF from epididymal WAT of PfnHet-HFD showed a markedly decreased ATM population within the CD45+ leucocyte pool (PfnHet 38.5 ± 5.0 vs. PfnWT 54.3 ± 6.2% of CD11b+, F4/80+, and CD45+ cells (Fig. 5A and B). Also, PfnHet fed NC exhibited decreased monocyte migration into the peritoneum and differentiation in response to thioglycollate as compared to PfnWT-NC (Supplementary Fig. 4). Additionally, PfnHet-HFD displayed a significant reduction of the frequency of CLS (defined as F4/80+ cells surrounding a degenerating adipocyte) compared with PfnWT-HFD (28 ± 3.4 vs. 49 ± 6.7 CLSs per 400 adipocytes) (Fig. 5C and D). In the same specimens, the mean adipocyte area was comparable in the two groups (7,650 ± 497 vs. 8,995 ± 762 μm²) (Fig. 5E). Although not identical, the distribution of frequency of adipocytes was not significantly affected by pfn haploinsufficiency for any size category (Fig. 5F). Together, these experiments indicate a critical function for pfn in diet-induced ATM infiltration and WAT remodeling.

**PfnHet show a M2-like ATM phenotype.** In addition to the difference in the absolute number of ATMs, we investigated whether pfn haploinsufficiency affected the phenotype of ATM subsets on HFD. ATMs, like other macrophages, exhibit a wide array of polarization states across the M1/M2 spectrum (6).

We found that ATMs from PfnHet had a significant percent decrease in the CD11c–MGL1– subset and a conspicuous switch toward CD11c+ MGL1+ compared with PfnWT (Fig. 6). In contrast, the expression of other markers of ATM activation such as MHC-II, Ly6C, and CD206 was not affected by pfn haploinsufficiency (Supplementary Fig. 5). These studies underscore that ATMs from PfnHet are biased toward an anti-inflammatory phenotype and implicate pfn as a potential key player in diet-induced ATM polarization.

**PfnHet mice exhibit decreased levels of systemic and WAT inflammatory mediators.** When assayed for prototypical mediators of inflammation, serum of PfnWT showed an HFD-induced increase in IL-1β (data not shown), tumor necrosis factor-α (TNF-α), IL-6, and chemokines CCL2 (MCP-1) and CXCL1, which contribute to the induction and progression of low-grade inflammation (34–36). These changes were largely prevented in PfnHet-HFD (Fig. 7A). Other chemokines were not affected by HFD or pfn haploinsufficiency (Fig. 7B). In addition, qRT-PCR from sorted ATMs reproduced the decrease in CXCL1 observed in the serum of PfnHet-HFD (Fig. 7C), whereas mRNA expression in the whole epididymal WAT mirrored the profile of such serum cytokines as CCL-2 (MCP-1), TNF-α, IL-10, and interferon-γ (IFN-γ) but not IL-6 (Fig. 7D). By contrast, adiponectin expression was unaltered in the WAT of the mice, suggesting that adiposity and adipocyte function are unaffected and the metabolic phenotypes of PfnHet mice are not explained by this adipokine (Supplementary Fig. 6).

**PfnHet have preserved Treg frequency.** The decline of Treg in the expanding WAT has been invoked as an early event in the disruption of adipose tissue immune homeostasis,
thus promoting inflammation and impaired insulin sensitivity (12–14). In keeping with this notion, the blunted inflammation and protection from diet-induced IR noted in PfnHet-HFD was accompanied by preserved frequency of Treg despite an unchanged number of CD4 and CD8 (PfnHet 25.9 ± 7.94 vs. PfnWT 19.1 ± 8.16) (Fig. 8).

DISCUSSION

The current study addressed the role of pfn in HFD-induced IR and inflammation. We have previously demonstrated that pfn is essential for atherosclerotic lesion formation (16) and that its protein levels are increased in human atheromas (22), in the aorta of diabetic rats, and in cultured ECs exposed to oxChol through recruitment of STAT3 to the pfn promoter (26). In the current study, we show that HFD also upregulates pfn mRNA expression in WAT and increased pfn protein levels both in adipocytes and in SMCs, which are enriched in ATMs. Another point of analogy with pfn regulation by oxChol was that HFD elicited STAT3 recruitment to the 5' UTR of pfn in SMCs in ex vivo ChIP experiments. These studies suggest that pfn overexpression may be triggered by inflammatory mediators activating STAT3 and involved in obesity and IR, such as IL-6 (35), and/or by oxChol moieties resulting from HFD feeding.

Of importance, PfnHet displayed near normalization of HFD-induced glucose intolerance in association with a dramatic reduction in ATMs and proinflammatory cytokines at both the systemic and the WAT level. How does attenuated pfn expression exert these protective effects? At least three lines of evidence indicate that reduced inflammation in WAT accounts in part for the blunted inflammation and the insulin sensitive phenotype of PfnHet at the systemic level. First, HFD resulted in pfn overexpression in WAT but not in liver and striate muscle (Fig. 1A), thus implying that HFD-mediated pfn elevation plays a role mainly in WAT. Second, the similarity of the cytokine profile in whole WAT and serum of PfnHet-HFD (Fig. 7) points to WAT as a primary source for these mediators in the circulation. Third, the preserved frequency of Treg in PfnHet indicates a role of pfn in the development and/or activation of these cells, which regulate WAT immune homeostasis as well as glucose tolerance at the systemic level (13,14). In this context, PfnHet SMCs exhibited increased expression of IL-10, which is synthesized by Treg and M2 ATMs, and can protect adipocytes from the detrimental effects of TNF-α on insulin sensitivity (7) and production of proinflammatory cytokines in response to TNF-α (13) that attract monocytes to WAT (36). In PfnHet-HFD, the decrease in the absolute number of ATMs (Fig. 5) and the switch toward resident CD11c-Mgl1+ ATMs (Fig. 6) are in keeping with cues generated from Treg that keep WAT inflammation in check despite HFD. Whether and how pfn levels directly influence the commitment of T-helper cells toward Treg is a question that is actively being investigated in our laboratory.

Pfn plays a central role in cytoskeleton dynamics because in addition to being essential for conversion of G-actin to F-actin (18,19), it can interact with a growing family of proline-rich proteins (37) and, therefore, can integrate actin-dependent and -independent signaling cues. Thus, one could speculate that pfn could affect glucose tolerance by modifying cycling of Glut-4 to the surface in insulin-dependent tissue. However, 3T3-L1 moderate overexpression of PfnWT (approximately threefold over empty vector-transfected cells) did not affect Glut-4 distribution within the cell either basally or on insulin stimulation (G.R.R., unpublished observations).
In addition to its important and well-characterized intracellular functions associated with actin binding, pfn has been found at low concentrations in the circulation. It is seen in rodent models of glomerulonephritis (38) and correlates with the severity of atherosclerosis in patients with coronary artery disease (22); it could mediate paracrine communications between adipocytes and immune cells in the WAT microenvironment or systemically between WAT and other tissues targeted by HFD (e.g., the liver).

Studies have underscored the role of pfn and the actin cytoskeleton in general in human disease. Mutations in the pfn gene were identified as a causative factor in a subset of patients with familial amyotrophic lateral sclerosis (39) in which a potential impact of inflammation is increasingly appreciated (40). Additionally, changes in actin cytoskeleton genes in striate muscle, driven by the serum responsive factor, have been linked to IR in patients with T2D and normoglycemic patients with a positive family history of T2D (41). Finally, circulating pfn levels significantly correlate with severity of atherosclerosis in patients with cardiovascular disease with and without T2D (22).

Our studies provide compelling evidence for a role of pfn in HFD-induced IR and inflammation likely resulting from modulation of immune homeostasis within the WAT microenvironment. The elucidation of the effect of pfn levels and function in subjects with IR and T2D will help to validate pfn as a pathogenetic factor and may pave the way for new treatments for these prevalent conditions.

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G.R.R. researched data, contributed to the experimental design and discussion, and wrote the manuscript. M.P. researched data, contributed to the discussion, and reviewed/edited the manuscript. D.E. researched data. J.L. and S.E.S. contributed to the experimental design and discussion and reviewed and edited the manuscript. G.R.R. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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