Netrin-1 promotes adipose tissue macrophage retention and insulin resistance in obesity

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During obesity, macrophage accumulation in adipose tissue propagates the chronic inflammation and insulin resistance associated with type 2 diabetes. The factors, however, that regulate the accrual of macrophages in adipose tissue are not well understood. Here we show that the neuroimmune guidance cue netrin-1 is highly expressed in obese but not lean adipose tissue of humans and mice, where it directs the retention of macrophages. Netrin-1, whose expression is induced in macrophages by the saturated fatty acid palmitate, acts via its receptor Unc5b to block their migration. In a mouse model of diet-induced obesity, we show that adipose tissue macrophages exhibit reduced migratory capacity, which can be restored by blocking netrin-1. Furthermore, hematopoietic deletion of Ntn1 facilitates adipose tissue macrophage emigration, reduces inflammation and improves insulin sensitivity. Collectively, these findings identify netrin-1 as a macrophage retention signal in adipose tissue during obesity that promotes chronic inflammation and insulin resistance.

Obesity and its comorbidities, type 2 diabetes and cardiovascular disease, continue to increase in incidence and are major threats to global health. Studies in mice and humans have shown that expansion of adipose tissue mass is closely associated with the recruitment of cells of the myeloid and lymphoid lineage1–7, which gives rise to a state of chronic inflammation. The accumulation of adipose tissue macrophages (ATMs) in obesity is considerable, with ATMs comprising up to 40% of visceral white adipose tissue (VAT)2. These cells secrete pro-inflammatory molecules, including tumor necrosis factor-α (TNF-α)8, interleukin-1β (IL-1β)9,10 and CCL2 (ref. 11), that contribute to both local and systemic inflammation, thus potentiating insulin resistance. The selective inhibition or genetic deficiency of factors that promote macrophage recruitment (for example, CCL2) or alter their inflammatory state (for example, IKK-β) reduces adipose tissue inflammation and insulin resistance in obese mice11,12. In human studies, the results of treatment of individuals with type 2 diabetes with the insulin-sensitizing thiazolidinediones showed a correlation between improved systemic insulin resistance and the reduction of ATMs and inflammatory factors13,14. These findings suggest that inflammation of the VAT compromises metabolic homeostasis.

Resident tissue macrophages are a heterogeneous population, and their tissue and function reflect their local metabolic and immune microenvironment. Macrophages that populate lean adipose tissue are thought to be similar to alternatively activated or M2 macrophages15, which characteristically secrete anti-inflammatory cytokines (for example, IL-10) and promote tissue remodeling. With overnutrition, increased numbers of classically activated or M1-like macrophages populate the VAT, where they secrete inflammatory factors that impair glucose homeostasis in this and other tissues16. Adipocyte-derived chemokines (for example, CCL2 (ref. 17) and leukotriene B4 (ref. 18)) and obesity-associated increases in lipolysis19 are thought to provoke this influx of inflammatory monocyte–derived macrophages. However, studies have shown that the M1 and M2 macrophage phenotypes are not firmly entrenched, and interventions that alter key signaling molecules controlling alternative activation, such as the peroxisome proliferator activated receptor-γ (encoded by Pparg), can regulate the dynamic balance of ATMs and insulin sensitivity20.

Despite recent advances in the understanding of the immune cell types that accumulate in adipose tissue with obesity, the underlying mechanisms that promote their accrual and sustain chronic inflammation are not well understood. We hypothesized that in addition to signals directing the recruitment of macrophages into adipose tissue, obesity provokes signals that promote macrophage retention. And as growing evidence supports roles for neuronal guidance molecules of the slit, semaphorin, ephrin and netrin families in regulating immune cell responses, including migration, adhesion and inflammatory status21–24, it is possible that such molecules have a role in the regulation of immunometabolism. Indeed, a recent study showed increased expression of semaphorin 3E in the VAT of obese mice and humans, where it promoted the recruitment and activation of macrophages25. Notably, such neuronal guidance cues act as both positive and negative regulators of cell migration.

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Netrin-1 is a secreted laminin-related molecule that orients axonal growth cones through both chemoattractive and chemorepulsive signaling. Netrin-1 achieves these opposing functions by engaging distinct receptors on its target cell: receptors of the DCC (deleted in colorectal carcinomas) family, including DCC and neogenin, mediate chemoattraction to netrin-1, whereas members of the UNC5 family (UNC5A–UNC5D) and the adenosine A2B receptor mediate chemorepulsion. Instructional roles for netrin-1 and its receptors have been demonstrated in organogenesis, angiogenesis, tumorigenesis and inflammation, suggesting that netrin-1 regulates cell migration in a broad context. Here we show that netrin-1 and its receptor UNC5B (Unc5b in mice) are highly expressed in obese but not lean adipose tissue of humans and mice and investigate the contribution of this guidance cue to high-fat diet (HFD)-induced inflammation and insulin resistance.

RESULTS

Obesity increases adipose expression of netrin-1 and Unc5b

To identify neuronal guidance cues regulated by obesity, we performed gene expression profiling of adipose tissue from C57BL/6 mice fed a standard chow or high-fat diet (60% kcal) for 20 weeks. Using a custom expression array comprising a complete panel of netrin, semaphorin, slit and ephrin guidance cues and their receptors, we found that netrin-1 (Ntn1) mRNA expression was higher in VAT from HFD-fed obese mice than in that from lean chow-fed mice (Fig. 1a and Supplementary Table 1). We confirmed that netrin-1 expression was higher in adipose tissue of HFD-fed compared to chow-fed mice by quantitative RT-PCR (qRT-PCR) (Fig. 1a) and western blotting (Fig. 1b) but found that serum netrin-1 levels were similar in both groups (Supplementary Fig. 1a). Furthermore, mRNA and protein levels of netrin-1 and its chemorepulsive receptor Unc5b were higher in adipose tissue of obese mice compared to lean mice, whereas expression of the chemoattractive receptors Dcc and Neo1 was similar between groups (Fig. 1a,b). The high expression of Ntn1 and Unc5b in adipose tissue was associated with high expression of the macrophage marker F4/80 (Emr1) and the proinflammatory cytokine genes Il6 and Tnf (Fig. 1a). In addition, the expression of other select neuroimmune guidance molecules was higher in adipose tissue of obese mice compared to lean mice, including of Sema3e (Fig. 1a), whose gene product was recently reported to promote monocyte recruitment into adipose tissue during HFD feeding.

To identify the cellular compartment expressing netrin-1 and Unc5b, we isolated mRNA from the adipocyte and stromal vascular fractions of VAT of chow- and HFD-fed mice and performed qRT-PCR analysis. Ntn1 and Unc5b mRNA levels were higher in the stromal vascular but not adipose tissue fraction of HFD-fed mice in comparison to that from chow-fed mice (Fig. 1c). Consistent with this, immunofluorescence staining of VAT from HFD-fed mice showed netrin-1 and Unc5b reactivity in crown-like structures that colocalized with staining for the macrophage antigen F4/80 (Fig. 1d). We did...
not detect netrin-1 and Unc5b staining in VAT from lean mice (data not shown), which indicates that this guidance cue–receptor pair is specifically expressed in macrophages that infiltrate obese VAT.

To investigate the translational relevance of our findings in mice, we measured the expression of netrin-1 and UNC5B in human adipose tissue from lean (body mass index (BMI) <25 kg/m²) and obese (BMI >28 kg/m²) individuals. NTN1 and UNC5H2 (the homolog of Unc5b) but not DCC mRNA levels were higher in adipose tissue from obese subjects compared to that from lean subjects, whereas mRNA expression for other neuronal guidance cues with immunoregulatory functions, such as SEMA3A, did not vary between obese and lean subjects (Fig. 2a). Immunofluorescence staining showed netrin-1 reactivity in adipose tissue from obese, but not lean, individuals that colocalized with CD68-positive macrophages in crown-like structures (Fig. 2b). Notably, netrin-1 serum concentrations were lower in obese (mean BMI 38.6 kg/m²) than in lean (mean BMI 18.2 kg/m²) individuals (Supplementary Fig. 1b), suggesting that netrin-1 is selectively increased in ATMs during obesity.

Palmitate regulates netrin-1 and Unc5b expression

We next sought to understand the mechanisms underlying ATM expression of netrin-1 and Unc5b during obesity. Serum free fatty acids (FFAs) are substantially elevated by HFD feeding and are important adipocyte-derived mediators of macrophage inflammatory responses.34,35 We tested whether palmitate, a saturated fatty acid that is abundant during obesity, could induce expression of netrin-1 and Unc5b in mouse bone marrow–derived macrophages (BMDMs). Ntn1 and Unc5b mRNA expression levels were five- and threefold...
higher, respectively, in BMDMs treated with palmitate conjugated to fatty acid–free BSA than in those treated with BSA alone (Fig. 3a). Furthermore, palmitate, but not BSA, induced the activity of luciferase reporter genes under control of the NTN1 and Unc5B promoters in human embryonic kidney (HEK) 293T cells; this activity was inhibited, in part, by pretreating cells with an inhibitor of nuclear factor-κB (NF-κB), BAY 11–7085 (Fig. 3b,c). Moreover, palmitate, but not BSA, induced secretion of netrin-1 by BMDMs, as measured by ELISA, and this secretion was inhibited by BAY 11–7085 (Fig. 3d).

As a number of studies have demonstrated paracrine interactions between adipocytes and macrophages in the white adipose tissue during inflammation, we next investigated whether adipocyte inflammatory factors secreted during obesity could induce ATMs from lean mice (Fig. 3e). We harvested conditioned medium from differentiated 3T3-L1 cells treated with palmitate and BSA and used this to treat BMDMs. Secreted factors from palmitate-treated but not BSA-treated adipocytes induced Ntn1 and Unc5b mRNA expression in BMDMs (Fig. 3e). As previously reported, palmitate-treated adipocytes expressed high levels of Tnf and Il6 mRNA (Fig. 3f), and treating BMDMs with TNF-α or IL-6, but not IL-4, increased Ntn1 mRNA expression (Fig. 3g). To test the role of these cytokines, we incubated conditioned medium from palmitate- and BSA-stimulated differentiated 3T3-L1 cells with blocking antibodies to TNF-α and IL-6, either alone or in combination, and used it to treat BMDMs. Antibodies to TNF-α or IL-6, but not control IgG, partially blocked the induction of Ntn1 and Unc5b mRNA expression by conditioned medium from palmitate-stimulated adipocytes (Fig. 3h and Supplementary Fig. 2), supporting a role for adipocyte-derived TNF-α and IL-6 in the induction of netrin-1 and Unc5b in neighboring macrophages.

Netrin-1 promotes defective ATM migration and accumulation

As netrin-1 has recently been reported to inhibit macrophage migration, we hypothesized that its secretion by ATMs may induce macrophage chemotaxis and block the egress of these cells from the VAT. To test this, we isolated CD11b+ F4/80+ ATMs from mice fed a standard chow diet or HFD for 16 weeks and measured their migration toward the chemokine CCL19, which has been implicated in the emigration of tissue macrophages to draining lymph nodes. Notably, ATMs isolated from obese mice exhibited less migration to CCL19 than did ATMs from lean mice (Fig. 4a), despite their equivalent expression of the CCL19 receptor Ccr7 (Fig. 4b). By contrast, blood monocytes isolated from lean and obese mice showed similar rates of chemotaxis to CCL2 (Supplementary Fig. 3a), consistent with reports that circulating monocytes from chow- and HFD-fed mice exhibit comparable recruitment to adipose tissue in vivo. Notably, ATMs from lean mice treated with netrin-1 showed less migration to CCL19 compared to untreated ATMs (Fig. 4c). Furthermore, ATMs from obese mice that had been incubated with a blocking antibody to Unc5b were able to migrate toward CCL19 to a similar degree as lean ATMs (Fig. 4d), which is consistent with a role for netrin-1 in the induction of ATM chemotaxis during obesity. Moreover, peritoneal macrophages treated with palmitate showed less migration to CCL19 than did BSA-treated macrophages (Fig. 4e). This reduced migration was restored by the addition of a chimeric Unc5b-Fc peptide that competitively

![Figure 4](https://example.com/figure4.png)
inhibits netrin-1, but not by addition of a control Fc (Fig. 4f). We observed similar findings when we used CCL2 as a chemoattractant (Supplementary Fig. 3b).

On the basis of these data, we postulated that ATM expression of netrin-1 during obesity may promote the accumulation of these cells in VAT and foster chronic inflammation and metabolic dysfunction. To test this, we generated mice with and without netrin-1 in macrophages by reconstituting the bone marrow of C57BL/6 mice with fetal liver cells from embryonic day 14 Ntn1+/− or Ntn1+/+ (wild-type WT) embryos and fed the chow or a HFD for 20 weeks. HFD-fed Ntn1+/−/C57BL/6 and WT→C57BL/6 mice gained more weight than their chow-fed counterparts; however, we observed no differences in food intake (Supplementary Fig. 4a) or body weight and fat content as measured by dual-energy X-ray absorptiometry (Fig. 4g,h) between the two genotypes. Notably, Ntn1+/−→C57BL/6 and WT→C57BL/6 mice fed a chow diet accumulated similar numbers of ATMs, whereas HFD-fed Ntn1+/−→C57BL/6 mice showed lower expression of Emr1 (F4/80) mRNA (Fig. 4i) and fewer F4/80+ cells in crown-like structures (Fig. 4j) in the VAT compared to HFD-fed WT→C57BL/6 mice. ATMs isolated from HFD-fed Ntn1+/−→C57BL/6 mice also had lower mRNA expression of proinflammatory cytokines and markers of M1 macrophages (Nos2, Tnf and Il6) and higher mRNA expression of markers of reparative M2 macrophages (Mrc1, Pparg and Il10) compared with HFD-fed WT→C57BL/6 mice (Fig. 5a). Thus, deletion of hematopoietic Ntn1 reduces ATM accumulation and restores the balance of M1 and M2 macrophages in obese adipose tissue.

To test whether netrin-1 affects macrophage mobilization into and out of VAT, we labeled monocytes in vivo with fluorescent beads and tracked them over time9. We measured macrophage recruitment and retention in WT→C57BL/6 and Ntn1+/−→C57BL/6 mice by comparing the number of bead-positive macrophages that accumulated in VAT 3 d after labeling (the time of peak recruitment of labeled cells to tissues9) and 11 d later to assess the number of labeled macrophages remaining. HFD feeding induced 2.5-fold greater recruitment of labeled monocytes into VAT compared to chow feeding in both WT→C57BL/6 and Ntn1+/−→C57BL/6 mice (Fig. 5b). The fluorescent beads carried in by these monocytes accumulated within F4/80-positive macrophages present in crown-like structures (Fig. 5c) to a similar extent in HFD-fed WT→C57BL/6 and Ntn1+/−→C57BL/6 mice after 3 d (Fig. 5b). However, the number of bead-labeled macrophages in VAT stayed constant in HFD-fed WT→C57BL/6 mice at day 14 (Fig. 5b,c), whereas there were 50% fewer bead-labeled macrophages in VAT of Ntn1+/−→C57BL/6 mice at this time point compared to day 3. As the reduction in bead-labeled macrophages in the Ntn1+/−→C57BL/6 mice at day 14 is indicative of ATM emigration from the VAT, we isolated the draining lymph nodes from the mesentery and analyzed the number of bead-labeled cells in this tissue. The mesenteric lymph nodes of HFD-fed Ntn1+/−→C57BL/6 mice contained 40% more bead-labeled cells compared to WT→C57BL/6 mice at day 14 (Fig. 5d and Supplementary Fig. 4b). Together, these data suggest that netrin-1 secreted by ATMs acts as a retention signal to promote ATM accumulation in the setting of obesity.

**Netrin-1 promotes insulin resistance in vivo**

The accumulation of macrophages within VAT has been shown to correlate with systemic inflammation and metabolic dysfunction, and consistent with this, we observed lower serum TNF-α concentrations in HFD-fed Ntn1+/−→C57BL/6 mice compared to HFD-fed WT→C57BL/6 mice (Fig. 6a). Furthermore, glucose and insulin tolerance tests revealed that HFD-fed Ntn1+/−→C57BL/6 mice had improved glucose homeostasis and insulin responsiveness compared to HFD-fed WT→C57BL/6 mice (Fig. 6b,c), as well as lower fasting blood glucose and triglycerides (Fig. 6d,e). Finally, we observed that glucose-stimulated insulin secretion was impaired in HFD-fed Ntn1+/−→C57BL/6 mice (Fig. 6f). Together, these data support the notion that netrin-1 promotes systemic inflammation and insulin resistance by promoting the accumulation of proinflammatory M1 macrophages within VAT.
(Fig. 6d), insulin (Fig. 6e) and FFA concentrations (Fig. 6f). Moreover, whereas plasma adiponectin concentrations were lower in HFD-fed WT→C57BL/6 mice compared to chow-fed mice, no difference was observed between HFD-fed and chow-fed Ntn1−/−→C57BL/6 mice (Fig. 6g). Finally, as the metabolic effects of insulin are dependent on phosphoinositide 3-kinase–AKT signaling in target tissues, we measured the phosphorylation of AKT on Ser473 in adipose tissue, liver and muscle. Notably, similar numbers of bead-labeled ATMs were detected in VAT of HFD-fed WT→C57BL/6 mice after 3 d and 14 d, indicating that ATMs recruited to adipose tissue of obese WT mice accrue in the tissue. By contrast, Ntn1−/−→C57BL/6 mice had 50% fewer bead-labeled ATMs in VAT and a greater number of bead-labeled cells in the mesenteric lymph nodes at day 14 compared to day 3, suggesting that ATMs emigrate from the VAT in the absence of netrin-1. These data suggest that during obesity, netrin-1 acts downstream of monocyte chemotactic signals, such as CCL2, to promote the local retention of ATMs in VAT. Indeed, targeted deletion of netrin-1 in hematopoietic cells induces a phenotype similar to that observed in Ccr2−/− mice: protection from HFD diet–induced adipose tissue inflammation and improved systemic glucose homeostasis and insulin sensitivity.

To understand how netrin-1 promotes ATM accumulation in obesity, we adapted a fluorescent bead monocyte-labeling technique that has been used to monitor the migration of macrophages into and out of atherosclerotic plaques. In vivo macrophage tracking demonstrated that HFD feeding induces recruitment of threefold more labeled macrophages into the VAT of both WT→C57BL/6 and Ntn1−/−→C57BL/6 mice compared to chow-fed mice of each genotype. Notably, similar numbers of bead-labeled ATMs were detected in VAT of HFD-fed WT→C57BL/6 mice after 3 d and 14 d, indicating that ATMs recruited to adipose tissue of obese WT mice accrue in the tissue. By contrast, Ntn1−/−→C57BL/6 mice had 50% fewer bead-labeled ATMs in VAT and a greater number of bead-labeled cells in the mesenteric lymph nodes at day 14 compared to day 3, suggesting that ATMs emigrate from the VAT in the absence of netrin-1. These data suggest that during obesity, netrin-1 acts downstream ofmonocyte chemoattractant signals, such as CCL2, to promote the local retention of ATMs in VAT. Indeed, targeted deletion of netrin-1 in hematopoietic cells induces a phenotype similar to that observed in Ccr2−/− mice: protection from HFD diet–induced adipose tissue inflammation and improved systemic glucose homeostasis and insulin sensitivity.

Studies in humans and animal models have shown that caloric restriction, or a switch from a high-fat to a low-fat diet, reduces adipose tissue inflammation. The signals controlling resolution of inflammation in the adipose tissue remain poorly defined but are likely to involve reduced recruitment, local macrophage death and efferocytosis, as well as egress of macrophages from the inflammatory site. In other sites, including the intestine and atherosclerotic plaques, the chemokine receptor CCR7 has been shown to promote the trafficking of macrophages to the lymph nodes. We show herein that ATMs isolated from obese but not lean mice exhibit impaired migration to the CCR7 ligand CCL19, despite their equivalent expression of CCR7. Notably, the responsiveness to CCL19 could be restored in obese ATMs by inhibiting the Unc5b receptor, suggesting that netrin-1 is responsible for this chemotaxis. Previous studies
have shown that netrin-1 inhibits Rac-mediated reorganization of the actin cytoskeleton and cell spreading, key steps required for cell migration\(^{35}\). Although the signaling pathways regulating netrin-1 expression in ATMs of obese mice and humans \textit{in vivo} will require further experimentation, previous studies have shown that increased FFA concentrations promote macrophage accumulation in the VAT\(^{19}\). Indeed, we demonstrate that palmitate directly induces \textit{Ntn1} and \textit{Unc5b} expression by macrophages and also upregulates expression of TNF-\(\alpha\) and IL-6 by neighboring adipocytes, which in turn can induce macrophage \textit{Ntn1} and \textit{Unc5b} expression.

Collectively, our studies suggest a model in which netrin-1 secreted by ATMs in the setting of obesity acts as a ‘stop’ signal to promote the accrual of these immune cells in the VAT. Macrophages, as a percent-age of total cells in the VAT, expand from ~10% in lean individuals to more than 50% in those with advanced obesity, yet the underlying cause and function of these ATMs remain poorly understood. A number of hypotheses regarding the triggers of ATM accumulation have been put forth, including an increase in adipocyte death in the expanding adipose tissue\(^{46}\), the release of saturated FFAs that activate inflammatory signaling pathways\(^{47,48}\) and the need for macrophages to buffer the lipids released from obese adipocytes in order to protect tissues from potentially toxic lipid levels\(^{49}\). These signals, either alone or combined, promote the sustained accumulation of ATMs and inflammatory molecules that impair the metabolic function of the VAT. We postulate that netrin-1 may be turned on as a signal to locally trap ATMs during obesity to help the VAT in the clearance of apoptotic adipocytes and the buffering of increased lipids; however, its sustained expression leads to chronic inflammation and the failure to reestablish tissue homeostasis.

It is notable that netrin-1 is selectively upregulated in the VAT, but not the serum, of obese mice and humans. In fact, we observed a modest but significant reduction in circulating levels of netrin-1 in obese individuals compared to lean controls and a similar trend in HFD-fed mice. Although the implications of these findings are unknown, recent studies have suggested that netrin-1 in the circulation may inhibit inflammation by reducing leukocyte recruitment into tissues. Netrin-1 is expressed on vascular endothelium and gut epithelium, where its expression is regulated to promote or inhibit leukocyte transmigration into tissues\(^{52,50–52}\). Reduced circulating levels of netrin-1 have been associated with neutrophil infiltration of the kidney during ischemia-reperfusion injury\(^{53,54}\). Furthermore, lentiviral overexpression of netrin-1 on endothelial cells protects \textit{Ldlr}\(^{-/}\) mice from atherosclerosis\(^{55}\), presumably by reducing leukocyte recruitment into the artery wall. However, netrin-1 is also abundantly expressed by macrophage foam cells in atherosclerotic plaques, where its expression promotes the accumulation of macrophages and disease progression\(^{33}\), consistent with our findings in obese VAT. Thus, therapeutic targeting of netrin-1 to reduce chronic inflammation in obesity and atherosclerosis will require macrophage-targeted delivery of netrin-1 or Unc5b inhibitors to avoid unwanted effects on netrin-1 at the endothelium or epithelium.

Current strategies aimed at targeting adipose tissue inflammation have so far yielded variable results: blocking TNF-\(\alpha\) signaling in obese individuals with type 2 diabetes produced suboptimal outcomes\(^{38,39}\), yet clinical trials involving inhibition of IL-1 signaling appear more promising\(^{37}\). However, these therapies directed at blocking cytokines that result from chronic inflammation are likely to be less effective than treatments targeting the source of this problem, i.e., the sustained accumulation of macrophages and other immune cells in VAT. Our work suggests that targeting local factors that promote the retention of macrophages, such as the netrin-1–Unc5b axis, may reverse the local and systemic mediators of inflammation that drive metabolic dysfunction.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

B.R., K.J.R. and K.J.M. conceived of the study; B.R. performed the \textit{in vivo} studies and data analyses, and assisted in the preparation of the manuscript; E.J.H., W.S. and S.H. assisted with mouse studies; F.J.S. performed ELISA assays; M.M. assisted with flow cytometry studies, S.O. performed RT-PCR; G.M. and R.M. collected human samples; K.J.R. and M.G. performed serum netrin-1 ELISA; T.D.R. performed western blotting; A.W. performed migration assays; and K.J.M. designed, analyzed and interpreted the studies, and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. 6- to 8-week-old male C57BL/6 mice and 26-week-old male C57BL/6 mice were fed a HFD consisting of 60% kcal from fat (D12492, Research Diets) or a control chow diet (P053, Picolab) for 20 weeks were purchased from Jackson Laboratories. All mice were maintained in a pathogen-free facility. For bone marrow transplantation studies, female and male Ntn1+/− mice (ten generations backcrossed onto C57BL/6 background) were mated to generate Ntn1+/− and Ntn1−/− donor mice. On day 14 of gestation, embryos were dissected free from the placenta and yolk sac, and single-cell suspensions of fetal liver cells were prepared by flushing through graded sizes of needles. A portion of the fetal tissue was used for genotyping by 5-bromo-4-chloro-3-indolyl-β-D-galactoside staining and PCR analysis. Fetal liver cells (2 × 10^6) were injected intravenously into 6- to 8-week-old recipient C57BL/6 mice that were lethally irradiated (two exposures of 600 cGy), and mice were allowed to recover for 6 weeks. C57BL/6 mice were fed a standard chow diet or HFD consisting of 60% kcal from fat (D12492, Research Diets) for 20 weeks. At the termination of the study, mice were anesthetized with tribromoethanol (0.4 mg per g body weight intraperitoneally (i.p.)) and exsanguinated by cardiac puncture. Experimental procedures were done in accordance with the US Department of Agriculture Animal Welfare Act and the US Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the New York University School of Medicine’s Institutional Animal Care and Use Committee.

Human subjects. Visceral adipose tissue (omentum and mesenteric) was collected from patients who underwent laparotomy for neoplastic disease at a site remote from the collection of visceral adipose tissue. Written informed consent was obtained from patients. The lean patients had a body mass index (BMI) ≤ 25 kg/m^2, Obese patients had a BMI ≥ 28 kg/m^2. The New York University School of Medicine Institutional Review Board approved tissue collection. Serum concentrations of netrin-1 were measured in a second group of healthy subjects [mean (71 ± 1.5 years)] lean subjects (BMI 18.2 ± 1.4 kg/m^2; n = 20; 6 male, 14 female) and obese patients (BMI 38.6 ± 2.4 kg/m^2; n = 20; 9 male, 11 female) recruited under Ottawa Hospital Research Ethics Board protocol #1997172-01H at the University of Ottawa Heart Institute.

Quantitative RT-PCR analysis. RNA (0.5–1 µg) was reverse-transcribed using iScriptTM cDNA Synthesis Kit (Bio-Rad), and RT-PCR analysis was conducted using KAPA SYBR green Supermix and a Mastercycler Realplex (Eppendorf). Data analysis was performed using the manufacturer’s integrated change calculations. RNA quality was verified by Pico Chips (5067–1511; Agilent Technologies).

Gene expression profiling. 1 mg of adipose tissue from either lean or obese animals was homogenized in TRIzol reagent (Invitrogen) using the Bullet Blender (New England Bio Group) and total RNA isolated as we previously described. Written informed consent was obtained from patients. The lean patients had a body mass index (BMI) ≤ 25 kg/m^2. Obese patients had a BMI ≥ 28 kg/m^2. The New York University School of Medicine Institutional Review Board approved tissue collection. Serum concentrations of netrin-1 were measured in a second group of healthy subjects [mean (71 ± 1.5 years)] lean subjects (BMI 18.2 ± 1.4 kg/m^2; n = 20; 6 male, 14 female) and obese patients (BMI 38.6 ± 2.4 kg/m^2; n = 20; 9 male, 11 female) recruited under Ottawa Hospital Research Ethics Board protocol #1997172-01H at the University of Ottawa Heart Institute.

Analytical procedures. Blood glucose values were determined from whole venous blood using an automatic glucose monitor (Freestyle Lite). The fat content in the mice was analyzed by dual-energy X-ray absorptiometry scanning. The mice were anesthetized and placed in the prone position on the specimen tray to allow scanning of the entire body. For food intake measurements, WT → C57BL/6 and Ntn1 → → C57BL/6 mice were fed a 60% kcal diet (D12492, Research Diets). Male mice were housed individually and were given a defined amount of large intact food pellet every 2 d. Food weight was measured using a balance with a precision of 0.01 g (OHAUS), and cages were changed. Solid food intake was corrected for any visible spillage.

Glucose and insulin tolerance tests. Glucose tolerance tests were performed as previously described. After determination of fasted blood glucose levels, each animal received a glucose gavage of 1.5 g per kg body weight of glucose (25% d-glucose, G7528; Sigma). Blood glucose levels were determined after 15, 30, 60 and 120 min. Insulin tolerance tests were carried out on unfasted animals by injecting an i.p. injection of 1.5 U per kg body weight of insulin (HumulinR 100 U/ml). Blood glucose levels were detected after 15, 30, 60, 120 and 240 min.

ELISA assays. Insulin (90080; Crystal Chem), adiponectin (ab108785; Abcam), FFA (SFA-1; Zen Bio), TNF-α (88-7064-76; ebioScience) and netrin-1 (E91827Mu; USCN Life Science) levels were measured in the serum or cell supernatants using mouse standards according to manufacturer’s guidelines. Netrin-1 in human serum was measured using a human-specific netrin-1 ELISA from USCN Life (E1910, Promega).

Labeling and tracking of blood monocytes. Circulating blood monocytes were labeled in WT → C57BL/6 and Ntn1 → → C57BL/6 mice fed a HFD for 20 weeks by retro-orbital intravascular injection of 1-µm Fluoresbrite green fluorescent plain microspheres (Polysciences) diluted 1:4 in sterile PBS as described.

Human subjects. Visceral adipose tissue (omentum and mesenteric) was collected from patients who underwent laparotomy for neoplastic disease at a site remote from the collection of visceral adipose tissue. Written informed consent was obtained from patients. The lean patients had a body mass index (BMI) ≤ 25 kg/m^2. Obese patients had a BMI ≥ 28 kg/m^2. The New York University School of Medicine Institutional Review Board approved tissue collection. Serum concentrations of netrin-1 were measured in a second group of healthy subjects [mean (71 ± 1.5 years)] lean subjects (BMI 18.2 ± 1.4 kg/m^2; n = 20; 6 male, 14 female) and obese patients (BMI 38.6 ± 2.4 kg/m^2; n = 20; 9 male, 11 female) recruited under Ottawa Hospital Research Ethics Board protocol #1997172-01H at the University of Ottawa Heart Institute.
Labeling efficiency was assessed 1 d after injection of beads. One group of mice was killed after 3 d for baseline measurements, as this time point has previously been shown to have optimal recruitment of labeled monocytes into tissues and clearance of the labeled monocytes from the blood. A second group of mice was killed 14 d later to measure the number of labeled macrophages remaining in adipose tissue. Adipose tissue and mesenteric lymph nodes were sectioned (5 µM), and the number of beads per section (×20) was counted from nine slides per mouse.

**Immunohistochemistry.** White adipose tissue was excised, fixed in formalin overnight, embedded in paraffin and sectioned. The immunofluorescence analyses of netrin-1 (1:200, AF1109; R&D Systems), caveolin-1 (1:500, 610059; BD Biosciences), Unc5b (1:200, ab54430; Abcam), F4/80 (1:500, MCA497GA; AbD Serotec) and CD68 (1:500, MCA1957; AbD Serotec) were conducted after deparaffinization as described previously. Secondary antibodies were then applied (AlexaFluor 568, A11011; AlexaFluor 488, A11006; 1:500; Invitrogen). Netrin-1 and Unc5b staining was amplified using biotin-conjugated antibodies (1:100, BA-2000 and BA-9500; Vector Laboratories) followed by streptavidin-conjugated AMCA (1:200, Sa-5008; Vector Laboratories) staining. Sections were mounted and visualized using a Nikon Eclipse microscope.

**Migration.** The migration of macrophages to CCL19 (500 ng/ml, 440-M3-025, R&D Systems) or CCL2 (100 ng/ml, 479-JE-010, R&D Systems) was assessed by the xCELLigence Real-Time Cell Invasion and Migration System (ACEA Biosciences), which monitored chemotaxis every 5 min for over 6 h. Peritoneal macrophages or BMDMs were pretreated with 250 µM palmitate or BSA before migration. The SVF was isolated from mice fed a chow diet (lean) or HFD (obese) for 14–16 weeks as described above, and the cells were sorted on magnetic columns to select for CD11b+F4/80+ (10 µl per 1 × 10⁷ cells, 130-049-601 and 130-099-440 for CD11b and F4/80, respectively, Miltenyi Biotec). In certain experiments, recombinant netrin-1 (250 ng/ml, 1109-NI-025, R&D Systems), rat Unc5b-Fc (1 µg/ml, 1006-UN-050, R&D Systems) or control IgG (110-HG-100, R&D Systems) was added to assay. Total blood was collected from either lean or obese mice, and the mononuclear cells were separated using Histopaque (10771, Sigma Aldrich) solution. After two PBS washes, the cells were counted and labeled using antibodies to CD11b (10 µl per 1 × 10⁷ cells, 130-049-601, Miltenyi Biotec) and Ly6G (10 µl per 1 × 10⁷ cells, 130-092-332, Miltenyi Biotec). CD11b+Ly6G− cells were separated using magnetic columns (130042201, Miltenyi Biotec) and then used for migration assays toward CCL2.

**Western blot analysis.** White adipose, liver and muscle tissues were homogenized in lysis buffer as previously described. Western blot analyses were carried out according to standard protocols with antibodies raised against netrin-1 (1:1,000, MAB1109; R&D Systems), Unc5b (1:1,000, AF1006; R&D Systems), AKT (1:1,000, 9272; Cell Signaling,) and phospho-AKT Ser473 (1:1,000, 9271; Cell Signaling). α-tubulin (1:5,000, T6074; Sigma) was used as loading control.

**Statistical analyses.** The difference between two groups was analyzed by Student’s t-test or for multiple comparisons, by one-way analysis of variance, followed by Newman-Keus multiple comparison test. P values of less than 0.05 were considered significant.

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Corrigendum: Netrin-1 promotes adipose tissue macrophage retention and insulin resistance in obesity

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In the version of this article initially published online, the corresponding author’s e-mail address was incorrect. The correct address is kathryn.moore@nyumc.org. The error has been corrected for all versions of this article.