Shining LIGHT on the metabolic role of the cytokine TNFSF14 and the implications on hepatic IL-6 production.

Bernadette Saunders¹, Caroline Rudnicka², Alexandra Filipovska³, Stefan Davies³, Natalie Ward⁴, Jana Hricova⁵, Markus Schlaich⁵,⁶, Vance Matthews⁵

¹School of Life Sciences, Faculty to Science University of Technology, Sydney, New South Wales, Australia.
²Research Centre, Royal Perth Hospital, Perth, Western Australia, Australia.
³Harry Perkins Institute of Medical Research, Nedlands, Western Australia, Australia.
⁴School of Biomedical Sciences, University of Western Australia, Perth, Western Australia, Australia.
⁵Dobney Hypertension Centre, School of Biomedical Sciences - Royal Perth Hospital Unit, University of Western Australia, Perth, Western Australia, Australia.
⁶Department of Cardiology and Department of Nephrology, Royal Perth Hospital, Perth, Western Australia, Australia.

CORRESPONDING AUTHOR:

Dr Vance Matthews,
SHORT TITLE: TNFSF14 and the metabolic syndrome.

KEYWORDS: TNFSF14, insulin resistance, obesity, diabetes, IL-6, liver

WORD COUNT: 4690
The cytokine Tumour Necrosis Factor Superfamily member 14, TNFSF14 (or LIGHT), is a controversial player in numerous diseases. We investigated the role of mouse TNFSF14 in diet-induced obesity in vivo. Specifically, we studied the effects of a global deletion of the TNFSF14 gene on the development of obesity, glucose intolerance, insulin resistance, steatosis, tissue inflammation and mitochondrial respiration in the liver. Secondly, we examined the role of TNFSF14 expression in hematopoietic cells on obesity and insulin sensitivity.

Male TNFSF14 knockout and wildtype mice were fed chow or high fat diet (HFD) for 12 weeks. In other experiments, wildtype mice were reconstituted with bone marrow cells from TNFSF14 KO mice and were fed chow or HFD for 12 weeks. All mice were metabolically phenotyped.

We show that HFD fed wildtype mice had elevated circulating levels of TNFSF14 in their serum. Liver and white adipose tissue are potential sources of this elevated TNFSF14. Excitingly, TNFSF14 deficient mice displayed markedly increased obesity, glucose intolerance, insulin resistance, hepatosteatosis and mitochondrial defects compared to wildtype mice on a HFD. Hepatic cytokine profiling pointed to a potential novel role of decreased IL-6 in the metabolic disturbances in obesogenic TNFSF14 KO mice. Finally, bone marrow cells from TNFSF14 deficient mice were able to contribute to promoting diet-induced obesity and insulin resistance.
Our novel data suggest that TNFSF14 ablation exacerbates parameters of the metabolic syndrome under high fat feeding conditions and provides further evidence to support the development of TNFSF14 agonists as potential therapeutics in diet-induced obesity.
INTRODUCTION

Global prevalence of obesity in adults has been significantly rising since the 1980s (Ng, et al. 2014) resulting in adverse metabolic conditions such as glucose intolerance, insulin resistance, inflammation, dyslipidemia and ultimately type 2 diabetes (T2D). Alarmingly, the cluster of pathologies characteristic of obesity-dependent type 2 diabetes, collectively referred to as ‘diabesity’, has started to emerge in children (Chen, et al. 2012; Farag and Gaballa 2011; Ng et al. 2014). This phenomenon warrants the urgent need to develop strategies to treat diabesity. Current diabesity treatment options initially focus on lifestyle modifications such as healthy diet, controlled caloric intake and increased physical exercise. Bariatric surgery is sometimes recommended (Colquitt, et al. 2014), although drug therapy is a viable intervention for those in whom lifestyle modification has failed (Li, et al. 2005; Sweeting, et al. 2015). Disappointingly, commonly used anti-obesity drugs have not resulted in consistent and effective weight loss (Li et al. 2005; Padwal and Majumdar 2007; Sweeting et al. 2015) and consequently there is great interest in developing new therapies to reduce obesity.

A number of secreted factors have been implicated in the etiology of obesity and insulin resistance in rodents and humans (Hotamisligil, et al. 1995; Hotamisligil, et al. 1993; Krogh-Madsen, et al. 2006; Steinberg, et al. 2006). One soluble factor of interest in alleviating the characteristics of diabesity is the Tumour Necrosis Factor Superfamily member TNFSF14 (also known as LIGHT; Lymphotoxin-like, exhibits Inducible expression, competes with Herpes Simplex Virus Glycoprotein D for Herpesvirus Entry Mediator [HVEM] receptor expressed by T lymphocytes). A number of studies have elegantly described a beneficial role
for TNFSF14 in numerous diseases. TNFSF14 has been shown to assist in wound healing (Dhall, et al. 2016), stem cell therapy (Heo, et al. 2016) and in skeletal muscle regeneration (Waldener-Streyer and Chen 2015). It is also reported that TNFSF14 serves a protective role in multiple sclerosis (Malmestrom, et al. 2013), experimental autoimmune encephalomyelitis (Mana, et al. 2013) and intestinal inflammation (Krause, et al. 2014).

Finally, a recent study presented that TNFSF14 is a potential immunotherapeutic agent to treat colon cancer (Qiao, et al. 2017).

Currently controversy surrounds the role of TNFSF14 in the development of the metabolic syndrome. Serum TNFSF14 levels are reportedly increased in morbidly obese humans (Bassols, et al. 2010a) and the expression of TNFSF14 is reduced in patients who do not have T2D compared with type 2 diabetic patients (Dandona, et al. 2014). However it remains to be elucidated whether TNFSF14 is functionally upregulated in the progression of the metabolic syndrome in a pro- or anti-obesogenic manner. Furthermore, the exact source of elevated TNFSF14 during the metabolic syndrome is unknown, although TNFSF14 is strongly expressed in multiple immune cells including resting and activated T cells, B cells, monocytes and macrophages (Kwon, et al. 1997). Adding complexity to this signalling cascade, TNFSF14, which may be expressed on the cell surface, secreted or cleaved by metalloproteinases, is considered a promiscuous ligand as it signals via the lymphotoxin-β receptor (LTβR) and herpesvirus entry mediator (HVEM). The HVEM receptor is highly expressed in visceral adipose tissue (Bassols, et al. 2010b) and both LTβR and HVEM are expressed in pancreatic β cells (Han and Wu 2009). Interestingly, treatment of human primary adipocytes with TNFSF14 resulted in a potent inhibition of adipocyte differentiation,
which suggests that TNFSF14 may be metabolically beneficial (Tiller, et al. 2011) although this remains to be comprehensively investigated.

We are the first research team to utilise a TNFSF14 knockout mouse to investigate the functional role of TNFSF14 in obesity, insulin signalling, inflammation, hepatosteatosis, cytokine signalling pathways and mitochondrial respiration. Secondly, our study determines the cellular sources of TNFSF14 that may attenuate diet-induced obesity. The outcomes of this study provide compelling evidence that TNFSF14 is necessary to limit the pathogenesis of the metabolic syndrome and our data supports the development of agonists of TNFSF14 signalling as attractive therapeutics for treating obesity and type 2 diabetes.
MATERIALS AND METHODS

Diet-induced obesity mouse model

Eight week old C57BL6/J mice were administered either a normal chow (chow) or high fat diet (HFD) (Speciality Feeds, Glen Forrest, WA Australia) for 12 weeks. Mice had free access to food and water. Body weights were measured weekly for all mice. Intraperitoneal glucose tolerance tests (GTT; 1 g/kg) and insulin tolerance tests (ITT; 0.5 U/kg) were performed in mice fasted for 6 hours on week 11 and 12 of the diet regimen respectively. After 12 weeks on their respective diets, mice were anaesthetised with methoxyflurane, underwent cardiac puncture to obtain blood and were euthanised by cervical dislocation. Tissues were collected and either fixed in paraformaldehyde and subsequently embedded in paraffin for immunohistochemistry experiments, frozen in OCT embedding medium for oil red staining, or snap-frozen and stored at -80°C for mRNA and protein expression studies. All animal experimentation was approved by the Royal Perth Hospital Animal Ethics Committee and were conducted in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation.

Tumour Necrosis Factor Superfamily Member 14 knockout mouse model

Tumour Necrosis Factor Superfamily Member 14 knockout (TNFSF14 KO) mice display no major developmental defects (Scheu, et al. 2002). The TNFSF14 mice were backcrossed 8 generations to C57BL6/J mice to reduce genetic heterogeneity. Eight week old male wildtype (WT) and TNFSF14 KO mice on a C57BL6/J background were administered either a normal chow or HFD (Speciality Feeds, Glen Forrest, WA, Australia) for 12 weeks. Mice were
weighed weekly and GTTs or ITTs were conducted and serum/tissue collection occurred as described previously.

**Bone marrow mouse model**

Using methodology previously described (Bollrath, et al. 2009; Ernst, et al. 2008) six week-old male C57BL6/J mice were lethally irradiated with two 5.5-Gy doses of gamma-irradiation from a $^{137}$Cs source (Gammacell 3000 Elan; MDS Nordion, Kanata, ON, Canada) separated by a 4-h interval. As the TNFSF14 KO mice are on a C57BL/6J background and express the alloantigen CD45.2 on their hematopoietic cells, we used congenic Pep3b B6 SJL/Ly5.1 mice (Animal Resources Centre, WA, Australia) as the WT counterparts because they express CD45.1 on their hematopoietic cells. This critically allowed distinction to be made between donor and recipient lymphocytes by flow cytometry. After the second dose of irradiation, WT mice were reconstituted with 2 million whole bone marrow cells from TNFSF14 KO mice and given drinking water containing antibiotics (1.1g/L neomycin sulfate and 1000U/L polymyxin B sulfate) for the first 2 weeks post-irradiation. Six weeks post-transplant, mice were administered either normal chow or HFD for 12 weeks. Mice were subjected to all of the aforementioned metabolic studies.

**Cell culture experiments**

L6 myoblast cells were purchased from the American Type Culture Collection (Manassas, Virginia, USA). Cells were cultured at 37°C, 5% CO2 in a humidified chamber. L6 myoblasts were seeded in 6 well culture plates and grown in low glucose Dulbecco’s Modified Eagle
Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin (Thermo Fisher, Melbourne, Victoria, Australia). Differentiation of the myoblasts was induced by transferring cells to medium containing 2% fetal calf serum when the myoblasts were ~90% confluent. Experimental treatments commenced after 7 days of differentiation when nearly all myoblasts had fused to form myotubes. Upon the day of experimentation, cells were serum starved for 4hrs. Media was changed to low glucose DMEM containing 4% fatty acid free BSA with palmitate (0.75 mM) to induce insulin resistance, or without palmitate, and cells were incubated for 24 hours. Cells were also treated with or without mouse TNFSF14 (100 ng/mL; Peprotech, Rocky Hill, USA) for the same 24hr incubation. Cells were then treated with or without insulin (250 ng/mL) for 30min before cells were lysed.

Confluent mouse pancreatic β cells, MIN6, were grown in high glucose (4.5 g/L) DMEM. Cells were treated with mouse TNFSF14 (200 ng/mL; Peprotech) for 48hrs. Media was then changed to low glucose (1 g/L) DMEM. After 45min, the low glucose DMEM was changed to high glucose (4.5 g/L) DMEM for 45min. Cell-free culture supernatant was collected and subjected to a mouse insulin ELISA (EZRMI-13K; Millipore, Australia).

**Gene expression assays**

RNA from murine tissue was extracted using Trizol reagent (Invitrogen, Thermo Fisher, Melbourne, Victoria, Australia) and cDNA synthesis was performed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Thermo Fisher, Melbourne, Victoria, Australia). Real-time PCR was performed to determine the mRNA abundance utilising a Rotor-gene real-
time PCR machine (Qiagen, Hilden, Germany) using pre-developed TaqMan probe (FAM labelled) and primer sets for HPRT (Mm01545399_m1); CXCL-10 (Mm00445234_m1); TNF-α (Mm00443260_g1) and TNFSF14 (Mm00619239_m1) (Applied Biosystems). Quantitation was conducted as previously described (Chan, et al. 2004).

Enzyme Linked Immunosorbant Assays

Serum was collected and analysed for circulating TNFSF14 levels as per manufacturer’s instructions (CSB-EL023991MO; Cusabio Biotech Co. Ltd, China). Serum was also analysed for circulating insulin using an insulin ELISA (EZRMI-13K; Millipore, Australia).

Liver tissue was collected and homogenised in cytosolic extraction buffer (10mM HEPES, 3mM MgCl2, 14mM KCl, 5% glycerol, 0.2% IPEGAL) containing phosphatase and protease inhibitors (Roche Diagnostics, Indianapolis, Indiana, USA). Protein concentration was quantified using protein assay solution (Bio-Rad, Hercules, California, USA). Protein lysates were analysed for IL-6, IL-10, IL-1β and IL-18 according to manufacturer’s instructions (ELISAKit.com, Scoresby, Victoria, Australia).

Western blotting

Rat L6 myotubes were lysed or murine liver tissue was homogenised using cytosolic extraction buffer containing phosphatase and protease inhibitors and protein concentration was quantified using protein assay solution (Bio-Rad, Hercules, California, USA). Protein lysates were solubilized in Laemmeli sample buffer and boiled for 10 min, resolved by SDS-PAGE on 10% polyacrylamide gels, transferred by semi-dry transfer to PVDF membrane and
then blocked with 5% milk powder. Membranes were incubated overnight in primary antibody [phospho-AKT Ser473 (9271; Cell Signalling Technology Inc, Danvers, Massachusetts, USA); hepatic Lipase H-70 (sc-21007; Santa Cruz Biotechnology Inc, Dallas, Texas, USA) or mouse anti-β actin antibody (ab6276; Abcam, Cambridge, UK)] using recommended dilutions. Membranes were washed 3 times and the appropriate secondary antibody was added to the membranes (GE Healthcare Australia, Parramatta, New South Wales, Australia). Detection of the relevant protein was performed via enhanced chemiluminescence (GE Healthcare) and visualised using a Multilmage II FC Light Cabinet (Alpha Innotech Corporation, San Leandro, California, USA). Densitometry was performed using the Alphalmager software (Alpha Innotech Corp.).

Haematoxylin and eosin staining

Mouse gonadal adipose tissue was dissected and fixed in 4% paraformaldehyde overnight before being incubated in 50% ethanol (by volume) and then promptly embedded with paraffin. Adipose tissue was cut into 5µM sections and stained with haematoxylin (Sigma-Aldrich, Sydney, New South Wales, Australia) and eosin (Sigma-Aldrich).

Oil red lipid staining

Freshly sectioned snap-frozen livers were fixed using 10% Formalin. Slides were then washed in 60% isopropanol and stained with Oil Red (O0625; Sigma-Aldrich) for 15 minutes. Slides were washed in 60% isopropanol and lightly stained with Harris Modified Hematoxylin.
Solution (HHS32-1L; Sigma-Aldrich). Slides were washed in water and mounted using gelatin/glycerol.

**Immunohistochemistry for hepatic lipase expression**

Paraffin embedded liver tissue was sectioned at 5µM onto slides and de-waxed in xylene and rehydrated in ethanol. Antigen retrieval was performed on the slides by heating in EDTA buffer (pH 8.5; Sigma-Aldrich). Endogenous peroxidases were blocked with 3% hydrogen peroxide solution and tissue was then blocked in 5% FCS. Sections were then incubated in hepatic lipase (H-70) antibody (sc-21007; Santa Cruz), followed by anti-rabbit antibody conjugated to horse-radish peroxidase (GE Healthcare), followed by treatment with diaminobenzidine (DAB; DAKO). Tissues were dehydrated in ethanol and xylene and mounted with DPX (Sigma-Aldrich).

**Hepatic mitochondrial respiration study**

Mouse liver mitochondria were isolated using a standard procedure involving homogenisation and differential centrifugation (Chappell and Hansford 1972). Mitochondrial respiration was measured using glutamate/malate, succinate/rotenone and ascorbate/TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride) according to Kuznetsov *et al.* (Kuznetsov, et al. 2008). Briefly, 80 µg of isolated mitochondria were resuspended in mitomedium B (0.5 mM EGTA, 3 mM MgCl₂, 20mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 1 g.l⁻¹ fatty acid-free BSA, 60 mM lactobionate, 110 mM mannitol, 0.3 mM...
DTT, pH 7.1 with KOH) and added to a 2 mL OROBOROS Oxygraph-2K Oxygen Electrode thermostatically maintained at 37°C.

**Statistical analysis**

All quantitative data are presented as mean ± or - SEM. A significance level of p<0.05 was considered significant. Significance was determined using 2-way ANOVA or Student t-tests. Graphs were generated using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA).
RESULTS

Serum TNFSF14 levels are elevated with diet-induced obesity.

We demonstrate that WT mice fed a high fat diet (HFD) possess elevated levels of circulating TNFSF14 protein (Figure 1A) as well as increased TNFSF14 mRNA expression in metabolically relevant tissues including white adipose tissue (Figure 1B) and liver (Figure 1C) compared with mice fed standard chow.

TNFSF14 protects against diet-induced obesity, glucose intolerance and insulin resistance.

To further elucidate the functional role of TNFSF14 in obesity and T2D, we compared TNFSF14 knockout (KO) to wildtype (WT) mice fed either standard chow or HFD. This mouse model allowed for the effects of endogenous TNFSF14 to be ascertained. There were no discernible differences in body weight for chow fed mice (Figure 2A). Interestingly, TNFSF14 KO mice were markedly more obese compared with their WT counterparts when placed on a HFD (Figure 2A). These results were observed in three independent experiments. This novel data suggests that the presence of TNFSF14 may attenuate diet-induced obesity. In addition, HFD-fed TNFSF14 KO mice were more glucose intolerant (Figure 2B) and insulin resistant (Figure 2C) compared with WT controls. Also, HFD-fed TNFSF14 KO mice developed hyperinsulinemia (Figure 2D). Taken together, these data support the notion that TNFSF14 expression is increased during the metabolic syndrome to work in a compensatory manner to limit diet-induced obesity and type 2 diabetes.

TNFSF14 reduces fatty acid induced insulin resistance in L6 myotubes.
Our *in vivo* findings provided an insight to explore the direct effect of TNFSF14 on insulin sensitivity in cells of one of the major metabolic tissue types, skeletal muscle. Impressively, TNFSF14 treatment promoted insulin sensitivity and overcame palmitate induced insulin resistance in L6 skeletal muscle cells (Figure 3A).

**TNFSF14 treatment promotes glucose stimulated insulin secretion from pancreatic beta cells.**

Using the pancreatic β cell line MIN6, we demonstrate that glucose stimulated insulin secretion is significantly elevated in response to TNFSF14 treatment compared with untreated cells (Figure 3B). Therefore, we show for the first time that insulin secretion may be directly stimulated in the presence of TNFSF14.

**Endogenous TNFSF14 expression reduces adipocyte hypertrophy and inflammation in white adipose tissue in HFD-fed obese mice.**

We then shifted our focus to investigating the role of TNFSF14 in metabolically relevant tissues. Firstly, we show that TNFSF14 deficiency promotes adipocyte hypertrophy under high-fat feeding conditions (Figure 4A, B). Furthermore, obesogenic TNFSF14 KO mice had significantly elevated mRNA levels of the pro-inflammatory cytokine TNF-α in their white adipose tissue compared with WT counterparts (Figure 4C) which indicates that TNFSF14 deficiency is associated with heightened inflammation.

**Hepatic lipid accumulation is promoted in HFD-fed TNFSF14 KO mice.**
We also assessed liver tissue from HFD-fed mice as pathological lipid accumulation is a hallmark of metabolic disease (Mehlem, et al. 2013). Oil red staining of liver sections indicated substantial increased lipid accumulation in the livers of TNFSF14 KO mice fed a HFD compared with WT mice (Figure 5A, B). Histological examination by haematoxylin and eosin staining further substantiated this. There was also elevated inflammatory cell infiltration and dilated vasculature in livers of TNFSF14 KO mice (Supplementary Figure 1). Combined, these data suggest an important role for TNFSF14 in lipid homeostasis.

Diet-induced obese TNFSF14 KO mice have elevated levels of hepatic lipase.

We hypothesised that there may be an association of TNFSF14 and lipases involved in lipid homeostasis. When we compared hepatic lipase protein expression in the livers of WT and TNFSF14 KO mice fed a HFD (Supplementary Figure 2), we unexpectedly observed elevated hepatic lipase protein expression in livers of TNFSF14 KO mice. We believe that this increase in hepatic lipase protein is a compensatory response to the high fat diet-induced steatosis.

Cytokine dysregulation in livers of TNFSF14 KO mice on a HFD.

We aimed to ascertain if TNFSF14 ablation triggers perturbations in cytokine expression in our diet-induced obese mice by assessing liver expression of cytokines which are known to be implicated in the pathogenesis of obesity and T2D. Interestingly, there was a trend for the chemokine CXCL-10 to be increased in livers from HFD-fed TNFSF14 deficient mice (Supplementary Figure 3A).
When we explored the effect of TNFSF14 on cytokine protein expression in the livers of HFD-fed mice, we saw a striking decrease in IL-6 expression in livers from TNFSF14 KO mice compared with WT counterparts (Figure 6A). In addition, the cytokine IL-10 was drastically upregulated in the livers of HFD-fed TNFSF14 KO mice compared with WT controls (Figure 6B). Although not significant, there was a trend for IL-1β (Supplementary Figure 3B) and IL-18 (Supplementary Figure 3D) protein expression to be reduced in livers from TNFSF14 KO mice compared to WT counterparts.

TNFSF14 deficient mice display dysregulated hepatic mitochondrial respiration when fed a HFD.

Defects in mitochondrial pathways can trigger metabolic changes such as obesity and insulin resistance (Baker, et al. 2014). Consequently we measured levels of mitochondrial respiration in the livers of HFD-fed mice. Levels of respiration using substrates for Complex I (Figure 7A), Complex II (Figure 7B) and Complex III (Figure 7C) were significantly upregulated in the livers of obesogenic TNFSF14 KO mice compared with WT mice. Our finding is consistent with a human study which reported that persons with steatosis have greater hepatic mitochondrial oxidative metabolism compared with controls (Sunny, et al. 2011). Hence it is plausible that TNFSF14 deficiency may contribute to hepatic mitochondrial defects under high fat feeding conditions.

Ablation of TNFSF14 in hematopoietically derived cells promotes diet-induced obesity and insulin resistance.
Given that hematopoietic cells are involved in diet-induced insulin resistance (Solinas, et al. 2007) and are a major source of TNFSF14, we sought to determine whether TNFSF14 deficiency in hematopoietic cells promoted diet-induced obesity and insulin resistance. We successfully demonstrate that hematopoietic cells are a major source of TNFSF14 that protects against diet-induced obesity as WT mice reconstituted with TNFSF14 KO bone marrow displayed significantly elevated diet-induced obesity (Figure 8A) and insulin resistance (Figure 8B) compared to WT mice reconstituted with WT bone marrow. Therefore we have now identified a major cellular source of the metabolically beneficial cytokine TNFSF14.
DISCUSSION

There is mounting evidence indicating the beneficial effects of the cytokine TNFSF14 in disease (Dhall et al. 2016; Heo et al. 2016; Krause et al. 2014; Malmestrom et al. 2013; Mana et al. 2013; Qiao et al. 2017). However, the role of TNFSF14 in the development of obesity and type 2 diabetes remains poorly understood (Bassols et al. 2010a; Dandona et al. 2014).

Our innovative study provides clear evidence that TNFSF14 is elevated in mice as an adaptive response to attenuate characteristics of the metabolic syndrome. To our knowledge, we are the first group to examine the functional role of naturally-expressed endogenous TNFSF14 in a murine model of diet-induced obesity. Mice globally deficient in TNFSF14 develop obesity, glucose intolerance and impaired insulin sensitivity under high-fat feeding conditions. Excitingly, we also show that lack of TNFSF14 leads to adipocyte hypertrophy and inflammation, hepatosteatosis and significant defects in hepatic mitochondrial respiration, indicating that TNFSF14 is required to hinder the development of complications arising from diet-induced obesity. Moreover, we show through in vitro experiments that TNFSF14 can overcome palmitate-induced insulin resistance in skeletal muscle cells and TNFSF14 treatment can directly promote insulin secretion from pancreatic β cells, signifying that TNFSF14 is metabolically beneficial in promoting insulin signaling and secretion. The work from our cellular studies is consistent with our finding that HFD-fed WT mice had improved insulin sensitivity compared with their TNFSF14 KO counterparts. Taken together, our novel data substantiates our hypothesis that physiological endogenous levels of the cytokine TNFSF14 are required for protection against features of the metabolic syndrome.
Cells of the hematopoietic lineage have been shown to play pivotal roles in diabesity (Solinas et al. 2007). As hematopoietic cells are a major source of TNFSF14, we sought to determine whether ablation of TNFSF14 in just the hematopoietic cells also promoted diet-induced obesity and insulin resistance. We hypothesized that if hematopoietic TNFSF14 is the major source of TNFSF14 that protects against diet-induced obesity and insulin resistance, then WT mice reconstituted with TNFSF14 KO bone marrow will display pronounced diet-induced obesity and insulin resistance when fed a HFD. If both hematopoietic and non-hematopoietic sources are involved, then the mice will display an intermediate phenotype. We conclusively show for the first time that hematopoietic cells are a source of protective TNFSF14 in our murine model of diet-induced obesity and T2D. It would be of interest to further determine the exact type of hematopoietic cell that is responsible for producing the metabolically beneficial cytokine TNFSF14, which include specific subsets of T cells, B cells or macrophages. It should be noted that the difference observed in weight gain and insulin resistance in our bone-marrow reconstitution experiments were smaller than those observed in our whole-body TNFSF14 knockout model. This suggests that hematopoietic cells are not the only source of protective TNFSF14 in diet-induced obesity and T2D. This is conceivable as we also demonstrated in our study that adipose tissue and liver are major sources of TNFSF14. Therefore adipocytes or hepatocytes may be candidate cells.

Hepatic lipase, an enzyme involved in lipid metabolism, hydrolyses triglycerides and phospholipids in lipoproteins and facilitates their metabolism and clearance (Santamarina-Fojo, et al. 2004; Teslovich, et al. 2010). Given the extent of chronic liver damage in
obesogenic TNFSF14 KO mice, we postulated that TNFSF14 deficiency may be associated with defects in hepatic lipase activity. We observed that HFD-fed mice lacking TNFSF14 had elevated hepatic lipase protein expression compared with their wildtype counterparts. This surprising result suggests that hepatic lipase may be potentially increased in the absence of TNFSF14 as an adaptive response to hydrolyse accumulated lipid in the liver (Chen, et al. 2015).

We next sought to determine whether mitochondrial respiration is influenced by the TNFSF14 KO phenotype in HFD fed mice. Liver mitochondria control hepatocellular energy metabolism via ATP synthesis and fatty acid oxidation (Pessayre, et al. 2002). It has previously been shown that insulin resistance is associated with impaired mitochondrial function in the liver (Kim, et al. 2008) though the role of mitochondrial function in disease is complex. In our study, we excitingly show for the first time that obesogenic TNFSF14 deficient mice exhibit a significantly elevated respiration rate when assessing respiration via Complex I, Complex II and Complex III, which we suggest is a compensatory attempt to prevent a decrease in ATP synthesis (Gonzalvez, et al. 2013) in the steatotic liver. Therefore, when mice are metabolically challenged with a HFD, TNFSF14 deficiency may be associated with dysregulated mitochondrial respiration in the liver.

The metabolically beneficial role of TNFSF14 in diet-induced obesity may also be associated with other factors which are potentially a direct result of the upregulation of TNFSF14. Hence, we conducted cytokine profiling in livers of HFD-fed mice to study the association of TNFSF14 with other cytokines known to be implicated in the metabolic syndrome. Of
particular interest are two interleukins, IL-6 and IL-10. Our group previously reported that IL-6 KO mice develop systemic insulin resistance and hepatic inflammation when fed a HFD (Matthews, et al. 2010). In agreement, HFD-fed IL-6 transgenic mice have lower body and fat mass, and are more glucose tolerant and insulin sensitive (Sadagurski, et al. 2010), suggesting that endogenous physiological levels of IL-6 may be beneficial in diet-induced obesity. Excitingly, we show for the first time that livers from obesogenic TNFSF14 KO mice have markedly lower hepatic IL-6 expression compared to livers from WT counterparts. The combined reduction of TNFSF14 and IL-6 expression may be one crucial accelerating factor implicated in the pathogenesis of obesity-induced liver disease. Indeed, the heightened level of IL-6 in WT liver supports the notion that IL-6 is metabolically beneficial in limiting HFD-induced steatosis. As some cellular studies have demonstrated that TNFSF14 directly induces IL-6 (Hosokawa, et al. 2010; Mikami, et al. 2014), it is intriguing to speculate that TNFSF14 directly regulates IL-6 production in our study.

We were also interested in the potential association of IL-10 and TNFSF14 as studies have previously reported that IL-10 is involved in the protection against diet-induced metabolic dysfunction including hyperinsulinemia (Grant, et al. 2014; Kesherwani, et al. 2015). Interestingly, mice fed a HFD and lacking TNFSF14 exhibited both hyperinsulinemia and significantly elevated expression of hepatic IL-10 compared with HFD fed WT mice. We believe that the increased hepatic IL-10 expression in the TNFSF14 KO mice on a HFD may be a compensatory mechanism to attempt to reduce hepatic inflammation as evidenced by the vast infiltration of inflammatory cells in the H&E stained liver sections of the TNFSF14 KO mice on a HFD.
Our study categorically indicates that endogenous physiological levels of the cytokine TNFSF14 has a protective role in the pathogenesis of obesity and type 2 diabetes and this may be facilitated by elevated hepatic IL-6 levels. Other studies have demonstrated that IL-6 behaves as a myokine during exercise to maintain glucose homeostasis (Febbraio, et al. 2004; Pedersen and Fischer 2007; Pedersen, et al. 2004). Indeed, muscle contraction during exercise appears to increase IL-6 production systemically which stimulates fatty acid oxidation and inhibits TNF-α induced insulin resistance (Pedersen and Fischer 2007). Therefore, it would be an intriguing future study to examine whether muscle derived IL-6 levels are also reduced in TNFSF14 KO mice fed a high fat diet.

A major novel aspect of our work is that we are one of the first groups to demonstrate circulating TNFSF14 levels using an in vivo mouse model. Until now, many studies have only assessed TNFSF14 levels by flow cytometry or mRNA levels of TNFSF14. Hence measuring TNFSF14 protein levels, as in our study, is highly relevant. We also feel that our knockout mouse model possesses a major benefit over studies which utilise transgenic mice. Our study examines the effects of endogenous TNFSF14 protein which exists at physiological levels. Unfortunately, studies utilising transgenic mice which overexpress cytokines produce supra-physiological levels of the protein of interest. For example, in the IL-6 setting, transgenic IL-6 mice express circulating IL-6 in the 3,000-15,000 pg/mL range (Benedetti, et al. 1997). Alternatively, endogenous levels of circulating IL-6 in many pathological conditions in mice only occur between 4-300 pg/mL (Das, et al. 2014; Han, et al. 2017; Wei, et al. 2015; Yeh, et al. 2011).
Our highly innovative study provides persuasive evidence that TNFSF14 expression is increased during the metabolic syndrome in a compensatory manner to reduce diet-induced obesity and T2D. Our exciting in vivo findings demonstrate an essential role for TNFSF14 in limiting high fat diet induced weight gain, glucose intolerance and insulin resistance. Furthermore, our data suggest a lack of TNFSF14 exacerbates chronic liver injury, inflammation and results in dysregulation of hepatic mitochondrial respiration. We also postulate that TNFSF14 may exert its protective effects in the liver via elevated IL-6 levels. Finally, we have shown for the first time that absence of TNFSF14 in bone marrow cells promotes obesity and insulin resistance. In conclusion, our novel data suggest that a TNFSF14 deficiency exacerbates parameters of the metabolic syndrome under high fat feeding conditions and provides further evidence to support the development of TNFSF14 agonists as potential therapeutics in diet-induced obesity.
DECLARATION OF INTEREST

The authors state that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Work described has not been published previously, it is not under consideration for publication elsewhere, its publication is approved by all authors, and if accepted, it will not be published elsewhere without the written consent of the copyright-holder.

FUNDING

The work described was generously funded by Diabetes Research WA, the Royal Perth Hospital Medical Research Foundation and a University of Western Australia (Safety Net Grant).

AUTHOR CONTRIBUTIONS

Bernadette Saunders conducted the breeding of mice, bone marrow transplantations, drafted the manuscript and funded the research. Caroline Rudnicka supervised, conducted experimental work and drafted the manuscript. Alexandra Filipovska conducted experimental work and drafted the manuscript. Stefan Davies and Jana Hricova conducted experimental work. Natalie Ward and Markus Schlaich drafted the manuscript. Vance Matthews funded, conceived, supervised, and conducted experimental work and drafted the manuscript.
ACKNOWLEDGEMENTS

The authors acknowledge the technical assistance of Ms Leah Stone and Ms Holly Allen.

REFERENCES


Waldemer-Streyer R & Chen J 2015 Myocyte-derived Tnfsf14 is a survival factor necessary for myoblast differentiation and skeletal muscle regeneration. *Cell Death Dis* **6** e2026.


**Figure 1.** TNFSF14 levels are increased in high fat fed mice. A high fat diet increases circulating TNFSF14 protein levels (A) and TNFSF14 mRNA expression in white adipose tissue (B) and liver (C); n=5-9 mice/group, *p<0.05, mean ± SEM.

**Figure 2.** TNFSF14 deficiency confers obesity, glucose intolerance, insulin resistance and hyperinsulinemia on a high fat diet. In a high fat feeding context, endogenous TNFSF14 ablation promotes obesity (A), glucose intolerance (B), insulin resistance (C) and elevated levels of circulating insulin (D). Glucose and insulin tolerance tests were conducted 12 weeks after high fat diet commencement; WT: wildtype, KO: TNFSF14 KO; n=14-26 mice/group, *p<0.05, **p=0.013, mean ± SEM.

**Figure 3.** TNFSF14 treatment reduces palmitate-induced insulin resistance and promotes insulin secretion in vitro. Representative immunoblot showing TNFSF14 treatment promotes insulin sensitivity in L6 skeletal muscle myotubes as indicated by increased expression of phospho-AKT. β-actin served as a housekeeping protein (A). Glucose stimulated insulin secretion from the MIN6 pancreatic β cell line is elevated after 48hrs of TNFSF14 treatment (B); n=3 wells/group, p<0.007, mean ± SEM.

**Figure 4.** TNFSF14 deficiency promotes adipocyte hypertrophy and inflammation in mice fed a high fat diet. Representative photomicroscopy depicting the reduced degree of
adipocyte hypertrophy in the white adipose tissue of WT mice (A) compared to TNFSF14 KO mice (B) on a high fat diet. Tnf-α mRNA expression is significantly increased in white adipose tissue of high fat fed TNFSF14 KO mice compared with WT counterparts (C); n=6-7 mice/group, *p<0.005, mean + SEM.

Figure 5. TNFSF14 deficiency promotes liver steatosis in mice fed a high fat diet.
Representative photomicrography showing oil red staining in livers of wildtype (A) and TNFSF14 KO (B) mice following 12 weeks of high fat feeding. Arrows indicate steatotic vesicles; n=8 mice/group.

Figure 6. Effect of TNFSF14 deficiency on cytokine expression in livers from high fat diet fed mice. TNFSF14 deficiency significantly reduces IL-6 expression (A) and increases IL-10 expression in livers from high fat diet fed mice; n=3-4 mice/group, *p=0.0091, **p=0.03139, mean + SEM.

Figure 7. TNFSF14 deficiency promotes mitochondrial compensation in livers of high fat diet fed mice. Respiration rates in liver mitochondrial homogenates when supplemented with substrates for either Complex I (A), Complex II (B) or Complex III (C); n=8 mice/group, *p<0.02, mean + SEM.

Figure 8. Hematopoietic cells from TNFSF14 KO mice promote high fat diet induced obesity and insulin resistance. Transfer of bone marrow from TNFSF14 KO mice into WT
mice promotes weight gain (A) and insulin resistance (B) in high fat fed mice. Weight analysis and insulin tolerance testing was conducted 12 weeks after commencement of high fat feeding; n=10 mice/group, *p<0.05, mean ± SEM.

Supplementary Figure 1. TNFSF14 deficiency promotes hepatic steatosis in high fat diet fed mice. Representative photomicrographs of hematoxylin and eosin staining of liver highlighting elevated lipid accumulation in the livers from WT (A) and TNFSF14 KO (B) mice.

Supplementary Figure 2. TNFSF14 deficiency results in a compensatory increase of hepatic lipase protein expression in high fat diet fed mice. Western blotting for hepatic lipase in WT and TNFSF14 KO mice fed a high fat diet (A). β-actin served as a housekeeping protein. Representative photomicrographs of hepatic lipase immunohistochemistry in the livers of WT (B) and TNFSF14 KO (C) mice fed a high fat diet-fed; 200x magnification; n=4 mice/group.

Supplementary Figure 3. Effect of TNFSF14 ablation on hepatic cytokine expression from mice on a high fat diet. CXCL-10 mRNA levels in the livers of WT and TNFSF14 KO mice fed a high fat diet for 12 weeks (A). There is a trend for lowered IL-1β (B) and IL-18 (C) in livers of TNFSF14 KO mice; n=3-8 mice/group, mean ± SEM.
Figure 2

A. % weight gain

B. Glucose (mmol/L)

C. Glucose (mmol/L)

D. Insulin (ng/mL)

Legend:
- WT HFD
- KO HFD
- WT CHOW
- KO CHOW

** and * indicate statistical significance.
Figure 3

**A.**

<table>
<thead>
<tr>
<th></th>
<th>+ vehicle</th>
<th>+ TNFSF14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin + Palmitate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B.**

![Insulin Graph]
Figure 8

A. % Weight gain over weeks for WT Bone Marrow to WT and KO Bone Marrow to WT.

B. Glucose levels in mmol/L over time for WT Bone Marrow to WT and KO Bone Marrow to WT.
Supplementary Figure 1

A. 

B. 

Supplementary Figure 2

A. 

WT HFD  |  KO HFD  |  Marker

Hepatic Lipase | 60 kDa  | 50 kDa
β-actin        | 40 kDa   

B. 

C. 

Supplementary Figure 3

A. CXCL10 mRNA expression (fold change relative to WT)

B. IL-1β (pg/mL per μg of total protein)

C. IL-18 (pg/mL per μg of total protein)