Introduction

Research over the last two decades has identified that chronic, low-grade inflammation plays a critical role in initiating insulin resistance, a key step in the development of type 2 diabetes [1]. Underpinning obesity-associated WAT inflammation is a marked shift in the immune cell profile of the WAT during the transition from a lean to obese state [2]. A loss of anti-inflammatory immune cells, concomitant with the accumulation of pro-inflammatory immune cells, initiates a state of local inflammation within the WAT that contributes to insulin resistance [2]. Studies in which particular immune cells or pro-inflammatory molecules have been genetically or pharmacologically targeted underscore the important role specific immune cells, and inflammation more generally, play in systemic insulin resistance [3–8].

Amongst the different classes of molecules shown to influence metabolism are protein tyrosine phosphatases [9]. The protein tyrosine phosphatase (PTP) superfamily is divided into classical PTPs and dual specificity phosphatases (DUSPs), the products of which catalyze the hydrolysis of phospho-tyrosine and phosphothreonine substrates [10]. Classical PTPs such as PTP1B and T-cell PTP have been identified as critical regulators of insulin and leptin signalling, serving important roles in the development of obesity and insulin resistance [11–14]. Of the many DUSPs described to date are the class I sub-family of DUSP proteins. The class I DUSPs specifically recognize and de-phosphorylate the conserved TxY motif present in the activation loop of the 4 major classes of mitogen-activated protein kinases (MAPK); ERK (1 and 2), ERK5, p38 (α, β, γ and δ) plus JNK (1, 2 and 3), and, accordingly, are also referred to as MAPK phosphatases (MKPs) [15]. Based on sequence homology, substrate specificity and subcellular localization, these MKPs can be further sub-divided into three groups. The first includes DUSP1/MKP-1, DUSP2

Abstract

Alterations in the immune cell profile and the induction of inflammation within adipose tissue are a hallmark of obesity in mice and humans. Dual-specificity phosphatase 2 (DUSP2) is widely expressed within the immune system and plays a key role promoting immune and inflammatory responses dependent on mitogen-activated protein kinase (MAPK) activity. We hypothesised that the absence of DUSP2 would protect mice against obesity-associated inflammation and insulin resistance. Accordingly, male and female littermate mice that are either wild-type (wt) or homozygous for a germ-line null mutation of the dusp2 gene (dusp2−/−) were fed either a standard chow diet (SCD) or high fat diet (HFD) for 12 weeks prior to metabolic phenotyping. Compared with mice fed the SCD, all mice consuming the HFD became obese, developed glucose intolerance and insulin resistance, and displayed increased macrophage recruitment and markers of inflammation in epididymal white adipose tissue. The absence of DUSP2, however, had no effect on the development of obesity or adipose tissue inflammation. Whole body insulin sensitivity in male mice was unaffected by an absence of DUSP2 in response to either the SCD or HFD; however, HFD-induced insulin resistance was slightly, but significantly, reduced in female dusp2−/− mice. In conclusion, DUSP2 plays no role in regulating obesity-associated inflammation and only a minor role in controlling insulin sensitivity following HFD in female, but not male, mice. These data indicate that rather than DUSP2 being a pan regulator of MAPK dependent immune cell mediated inflammation, it appears to differentially regulate inflammatory responses that have a MAPK component.

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* Email: Graeme.Lancaster@bakeridi.edu.au
† MAF and SG are joint senior authors on this work.
Bone marrow-derived macrophages (BMDM) were generated from the hind limb bones of *dusp2*<sup>−/−</sup> and *wt* C57Bl/6J mice. After 7 days of culture in RPMI media containing 15% fetal bovine serum (FBS) and 20% L-cell conditioned media, BMDM were changed overnight to RPMI media containing 5% FBS and 2% bovine serum albumin (BSA) prior to experimental treatment. Palmitate (0.500; Sigma Aldrich) was dissolved in 100% ethanol at a stock concentration of 100 mM and was then conjugated to bovine serum albumin (BSA) (2% weight per volume; A6003; Sigma Aldrich) in RPMI media containing 5% FBS to create a final concentrations of 1 mM. The vehicle contained BSA and ethanol, but no palmitate. Media was collected after 8 h of treatment with either palmitate or vehicle and the concentrations of the pro-inflammatory cytokines IL-1β and TNFα were then determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, USA). For the analysis of intracellular signals, cells were lysed after 4 h of treatment with palmitate or vehicle, protein concentrations determined and samples examined by Western blotting as described below.

Mice were administered 2 g of glucose per kg of lean mass by oral gavage following a 5 h fast to assess glucose tolerance. Blood was obtained prior to and at the indicated intervals for the proceeding 2 h from the tip of the tail. Blood glucose concentrations were determined using a glucometer (Accu-Check, Roche, NSW, Australia). Fat and lean mass were determined using an EchoMRI 4-in-1 (Echo Medical Systems, Houston, TX, USA). Plasma insulin concentrations were determined using a mouse ultrasensitive plasma insulin ELISA (ALPCO, Salem, NH, USA).

For quantitative RT-PCR, RNA was extracted from epididymal WAT using Trizol (Invitrogen, Carlsbad, CA, USA) and total RNA measured using the ND-1000 NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA samples were treated with DNase I (Invitrogen) and cDNA generated using the Tetro cDNA synthesis kit (Bioline). Gene expression studies were performed by RT-PCR using TaqMan primers and probes for genes of interest and 18S rRNA (Applied Biosystems, Foster City, CA, USA). For cytokine and *dusp2* gene expression, 40 ng of cDNA was used, whereas *f4/80* and *cd11c* expression was determined using 20 ng of cDNA. The comparative Ct method was used to quantify results from RT-PCR.

Genotyping of tail or ear clip DNA samples was performed using the following primers: WT: Forward - 5′-ATTGTGCTCTC-CCTTCTCTGGA-3′ and Reverse 5′-TGACACACACGCT-CACCTTCT-3′. *dusp2 KO*: Forward 5′-ATTGTGCTCTCC-TTCTCTGGA-3′ and Reverse 5′-CGCTGCTCTTACTGGA-GGC-3′. The following cycling conditions were used: 1 cycle of 3 min at 95°C, 60 s at 57°C, 45 s at 72°C, followed by 39 cycles of 60 s at 95°C, 60 s at 57°C, 45 s at 72°C. Products were resolved on a 2% agarose gel containing ethidium bromide.

For Western blotting, epididymal WAT was removed from mice under anesthesia, snap frozen in liquid N<sub>2</sub> and stored at -80°C. WAT was lysed and protein concentrations determined using the BCA method (Thermo Scientific). Samples (20 ug total protein per sample) were solubilized in Laemmli’s buffer and heated at 95°C for 5 min, then western blotting performed as described previously [23]. Antibodies against phosphorylated (4671) and total (9258) forms of JNK, phosphorylated (9211) and total (9212) forms of p38, and total ERK1/2 (9102) were from Cell Signalling Technology Inc (Danvers, MA, USA). The antibody against phosphorylated ERK1/2 (sc-7383) was from Santa-Cruz Biotechnology, Inc.

Statistical analysis was performed using SigmaStat version 3.5. Statistical significance was determined using a Student’s t-test or 2 way ANOVA analysis, where appropriate. Data are presented as
the mean ± the standard deviation. A p-value of less than 0.05 was considered statistically significant; although we have indicated where the p-values were lower than 0.05. Quantification of western blots was done using Quantity One (Bio-Rad Laboratories).

Results

DUSP2 expression is markedly increased in many immune cell types following activation [18,22]. Given activated immune cells accumulate in the adipose tissue of obese mice, we first determined whether dusp2 gene expression was increased in the epididymal WAT of obese mice. Consistent with the links between immune cell dependent inflammation and obesity, dusp2 mRNA expression was significantly higher in WAT from wt mice fed a HFD compared with wt mice fed a SCD (Figure 1).

To investigate the role of DUSP2 in the context of obesity-associated inflammation and insulin resistance, we exploited a mutant strain of mice that lack DUSP2 [18]. Male and female wt and dusp2−/− litter mates were fed either a SCD or HFD for 12 weeks commencing at 8 weeks of age. DUSP2-deficient mice have no known lymphoid or myeloid developmental abnormalities and have normal longevity free from any spontaneous immunopathology [18]. Both male (Figure 2a–c) and female (Figure 2d–f) wt and dusp2−/− mice responded as expected to the HFD. The HFD resulted in an increase in total body mass (Figure 2a and d), an effect entirely attributable to an increase in fat mass (Figure 2b and e). While male mice appeared to gain fat mass more rapidly than female mice fed a HFD, at the end of the dietary period both male and female mice, irrespective of genotype, had gained similar amounts of fat mass (Figure 2b and e).

To assess glucose tolerance and insulin sensitivity, we performed oral glucose tolerance tests (OGTT) and assessed plasma insulin levels during the OGTT in both male and female mice after 4 and 12 weeks of either SCD or HFD. Compared with those fed the SCD, male mice fed the HFD were glucose intolerant (Figure 3a and d), while DUSP1 expression was increased in both male and female mice following the HFD we observed no compensatory increases while DUSP1 expression was increased in both male and female mice. Given the published data ascribing an important role to DUSP2 in promoting inflammation and immune cell activation, we next investigated whether the absence of DUSP2 influenced epididymal adipose tissue inflammation. Total macrophage numbers, which included the recruitment of pro-inflammatory macrophages, inferred from the levels of f480 and cd11c gene expression, were markedly elevated following 12 weeks of HFD (Figure 5a and b). Consistent with this finding, levels of gene expression for monocyte chemo-attractant protein 1 (mcp1), the pro-inflammatory cytokines tumour necrosis factor (tnf) and interleukin 1β (il1β), plus the anti-inflammatory cytokine il10, were all significantly higher following 12 weeks of HFD (Figure 5c–f), emphasizing the potent pro-inflammatory environment in adipose tissue induced by obesity. Although the HFD led to significant increases in the expression of f480, cd11c, tnf, il1β and mcp1 in female mice (Figures 5a–e), these differences were markedly lower than those observed in male mice. Notably, an absence of DUSP2 did not affect macrophage markers, pro-inflammatory cytokine or il10 expression in male mice (Figures 5a–f). A near identical expression pattern for these genes was observed in female mice, the exception being il10, for which a small, but significant genotype-dependent difference was observed (Figure 5f). Furthermore, with the exception of il1β, the recruitment of macrophages and the increases in pro-inflammatory gene expression were markedly lower in female compared with male mice. This trend is consistent with the known anti-inflammatory effects of estrogen [24] and the finding that a loss of estrogen receptor α expression in the myeloid cells of female mice exacerbates obesity-associated WAT inflammation [25]. Finally, while DUSP1 expression was increased in both male and female mice following the HFD we observed no compensatory increases in Dusp2 expression in epididymal WAT of either male or female dusp2−/− mice (Figure 5g).

The activation of JNK and subsequent serine phosphorylation of insulin receptor substrate 1 (IRS1) in adipose tissue is postulated to be a key nexus by which a number of obesity-associated stimuli can directly impair insulin-dependent signaling [1]. A report that JNK phosphorylation is abnormally elevated in dusp2−/− cells following stimulation [18] prompted us to examine the impact of DUSP2 on JNK activation in epididymal adipose tissue. We observed a significant increase in the level of JNK phosphorylation in the WAT of male mice following 12 weeks of HFD (Figure 6a). However, the absence of DUSP2 had no additional impact on the levels of JNK phosphorylation in WAT (Figure 6a). We also determined ERK1/2 (Figure 6b) and p38 (Figure 6c) phosphorylation levels in WAT by western blotting. Neither the HFD nor DUSP2 deletion affected ERK1/2 or p38 phosphorylation in WAT.

A loss of DUSP2 activity in immune cells was previously reported to reduce LPS-induced pro-inflammatory cytokine production, but potentiate LPS-induced JNK phosphorylation [18]. Accordingly, we investigated whether pro-inflammatory cytokine production and JNK activation in BMDM activated by the long chain saturated fatty acid palmitate was also affected by an absence of DUSP2. While palmitate treatment resulted in increased TNF and IL-1β secretion as well as elevated JNK

![Figure 1. HFD increases dusp2 mRNA expression.](https://example.com/figure1.png)
Figure 2. The development of obesity in wt and dusp2−/− male and female mice. (a and d) Total body mass; (b and e) fat mass; and (c and f) lean mass in wt and dusp2−/− male and female mice fed either a SCD or HFD for 12 weeks. Data are mean ± SD. White squares are wt mice fed SCD; Black squares are dusp2−/− mice fed SCD; White circles are wt mice fed HFD; Black circles are dusp2−/− mice fed HFD. For male mice, Ns are 5, 8, 8 and 8, for wt SCD, dusp2−/− SCD, wt HFD and dusp2−/− HFD, respectively. For female mice Ns are 9, 8, 7 and 7 for wt SCD, dusp2−/− SCD, wt HFD and dusp2−/− HFD, respectively.

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Figure 3. Glucose tolerance and insulin sensitivity in wt and dusp2−/− male mice after 4 and 12 weeks of diet. (a and d) oral glucose tolerance; (b and e) plasma insulin during the OGTT; and (c and f) plasma insulin area under the curve (AUC). *p<0.05; Main Effect for HFD vs SCD. Data are mean ± SD. White squares are wt mice fed SCD; Black squares are dusp2−/− mice fed SCD; White circles are wt mice fed HFD; Black circles are dusp2−/− mice fed HFD. Ns are 5, 8, 8 and 8, for wt SCD, dusp2−/− SCD, wt HFD and dusp2−/− HFD, respectively.

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phosphorylation, we observed no differences in these responses between wt and dusp2−/− BMDM (Figure 7a–d).

Discussion

Studies reporting roles for the MAPK dual specificity phosphatases DUSP1 and DUSP9 during inflammation, obesity and insulin sensitivity, prompted us to investigate what function DUSP2 might play in these processes. DUSP2, a nuclear specific DUSP that is mainly expressed in immune cells, had been shown in macrophages and mast cells to promote the production of various inflammatory mediators [18] that included key mediators of obesity-induced metabolic disease. Based on these properties of DUSP2, we examined whether obesity driven inflammation in WAT and its associated impact on glucose intolerance and insulin resistance would be reduced in mice lacking DUSP2. In contrast to the previously ascribed role for DUSP2 regulating inflammatory responses in culture and in vivo, our findings reported here demonstrate that DUSP2 plays no role in the development of obesity or obesity-associated inflammation. Furthermore, a lack of DUSP2 activity does not prevent the development of obesity-associated glucose intolerance or insulin resistance in male mice, although it does appear to have a small protective effect in female mice.

Previous studies have demonstrated roles for DUSP family members in the development of obesity, and obesity-associated inflammation and insulin sensitivity. DUSP1-deficient (dusp1−/−) mice, which exhibit elevated MAPK activity in several metabolically important tissues including skeletal muscle, liver and adipose tissue [20] have significantly less fat mass than control animals when fed a HFD for 12 weeks [20]. Nevertheless, they are still not protected from the deleterious effects a HFD has on glucose metabolism [20]. DUSP9 over-expression in genetically obese ob/ob mice on the other hand, improved glucose tolerance [19], while DUSP9 over-expression in 3T3-L1 adipocytes suppressed ERK1/2 and JNK activation, with a concomitant reduction in IRS1 serine phosphorylation that prevented the induction of insulin resistance [19]. A comparative evaluation of the biochemical properties of DUSP1 and DUSP9 with those of DUSP2, in conjunction with the impact their loss of function or over-expression has on obesity, inflammation and insulin sensitivity, provides valuable insight into the different mechanisms by which certain DUSPs, but not others, appear to regulate metabolism. For example, both DUSP1 and DUSP2, which are localized to the nucleus, are induced by stress or mitogenic signals and share overlapping substrate specificities for ERK and p38 [26], have different impacts on whole body metabolism, with DUSP1, but not DUSP2, controlling fat mass. This difference in the regulation of fat mass may reflect the broader pattern of DUSP1 expression and the ability of DUSP1 to also dephosphorylate JNK [27]. Although DUSP2 and DUSP9 can both dephosphorylate ERK, these DUSPs are restricted to the nucleus and cytoplasm respectively [27]. In the context of inflammation-induced insulin resistance seen in obesity, the subcellular localization of these DUSPs may be a critical point of difference, with serine phosphorylation of IRS1 by various serine kinases including ERK1/2 and JNK thought to be a key effector mechanism by which pro-inflammatory stimuli induce cellular insulin resistance [1]. As the phosphorylation of IRS1 occurs at the plasma membrane, IRS1 is both a key mediator of cellular insulin resistance and a target for the development of insulin resistance in obesity. The altered subcellular localization of DUSP2 and DUSP9 may thus contribute to the different effects these DUSPs have on whole body metabolism. While it may be that DUSP2 and DUSP9 have different targets in WAT and other tissues, where these DUSPs are expressed, this remains to be explored. For example, DUSP2 has been shown to dephosphorylate JNK in vitro [27], and previous studies have shown that JNK is induced in obesity [28] and plays an important role in the development of insulin resistance through the regulation of protein kinase C theta (PKCθ) activity [29]. It is possible that the lack of a role for DUSP2 in the regulation of obesity and insulin resistance seen in our study may be due to a lack of activity in WAT. It is also possible that DUSP2 plays a role in the regulation of insulin resistance in other tissues, which are not covered by our study, such as skeletal muscle.

Figure 4. Glucose tolerance and insulin sensitivity in wt and dusp2−/− female mice. (a and d) oral glucose tolerance; (b and e) plasma insulin during the OGTT; and (c and f) plasma insulin area under the curve (AUC). *p<0.05; Interaction Effect wt HFD vs. dusp2−/− HFD. † p<0.05 Interaction Effect wt SCD vs. wt HFD and dusp2−/− SCD vs. dusp2−/− HFD. Data are mean ± SD. White squares are wt mice fed SCD; Black squares are dusp2−/− mice fed SCD; White circles are wt mice fed HFD; Black circles are dusp2−/− mice fed HFD. Ns are 9, 8, 7 and 7 at 4 weeks and 9, 7, 6 and 7 at 12 weeks for wt SCD, dusp2−/− SCD, wt HFD and dusp2−/− HFD, respectively.

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membrane, it is DUSP9 rather than DUSP2 that has access to cytoplasmic ERK.

Given the importance of inflammation and the accumulation of numerous immune cell types in the WAT of obese mice, we hypothesized that a loss of DUSP2 function might reduce obesity-associated macrophage recruitment or inflammation in WAT. However, unlike the previous role ascribed to DUSP2 in positively regulating inflammatory responses, our data reveal that DUSP2 has no effect on obesity-associated inflammation. Several explanations could account for these differences in the roles of DUSP2 in vivo. For example, the qualitative and/or quantitative features of an inflammatory response may be important in dictating the involvement of DUSP2. Whereas DUSP2 was found to promote pathology in an arthritis model that rapidly induces leukocyte recruitment and pronounced inflammation within several days [18], obesity-associated inflammation in mice, by contrast, often takes weeks to months to manifest and is typically a low grade inflammatory response. Therefore, it is possible that the low-grade inflammation associated with obesity is not a sufficiently strong stimulus to employ DUSP2 as a means of modulating MAPK dependent inflammatory responses. Indeed, this may be reflected in the relatively modest increase in DUSP2 expression in the WAT of mice fed a HFD. Functional redundancy amongst the nuclear specific DUSPs, which in addition to DUSP2 include DUSP1, DUSP4 and DUSP5, may also be a contributing factor in DUSP2 not playing a unique role in obesity-associated WAT inflamma-

Figure 5. Inflammation in the WAT of wt and dusp2−/− male and female mice. The mRNA expression of (a) f480; (b) cd11c; (c) mcp1; (d) tnf; (e) il1b; (f) il10 and (g) dusp1 in epididymal adipose tissue. *p<0.05 Main Effect SCD vs. HFD; **p<0.01 Main Effect SCD vs. HFD; ***p<0.005 Main Effect SCD vs. HFD; †p<0.05 Main Effect wt vs. dusp2−/−. Data are mean ± SD. Black bars are male mice, white bars are female mice. Ns are 5, 8, 8 and 8 for male mice and Ns are 8, 8, 6 and 7 for female mice for wt SCD, dusp2−/− SCD, wt HFD and dusp2−/− HFD, respectively.
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Not only does DUSP2 share overlapping substrate specificity for MAPKs with certain of these other nuclear DUSPs [26,27], it also has a similar pattern of expression with other nuclear DUSPs in activated leukocytes, in particular DUSP4 and DUSP5 [18]. Support for functional redundancy amongst the nuclear DUSPs comes from the recent report showing that in contrast to mice lacking either DUSP1 or DUSP4, which exhibit no cardiac pathology, mice missing both of these DUSPs develop cardiomyopathy due to unrestrained p38 activation [28].

Figure 6. MAPK analysis in epididymal adipose tissue. JNK (a), ERK (b) and p38 (c) phosphorylation status in epididymal adipose tissue of male mice. ***p<0.005 Main Effect SCD vs. HFD. Data are mean ± SD. White bars are wt mice, black bars are dusp2<sup>−/−</sup> mice. Ns are 5, 7, 8 and 8 for wt SCD, dusp2<sup>−/−</sup> SCD, wt HFD and dusp2<sup>−/−</sup> HFD, respectively. doi:10.1371/journal.pone.0111524.g006

Figure 7. Treatment of BMDM from wt and dusp2<sup>−/−</sup> mice with the free fatty acid palmitate. BMDM were treated for 8 h (a and b) or 4 h (c and d) with either a control stimulus (BSA; white bars) or 1 mM Palmitate (black bars). d shows representative western blots from the quantifications shown in c. Data represent 3 separate mice per genotype each performed with 3 technical replicates. Data are mean ± SD. doi:10.1371/journal.pone.0111524.g007
While the absence of DUSP2 had no impact on obesity-associated inflammation and insulin resistance in male mice, we did observe a small, but significant improvement in the insulin sensitivity of female DUSP2-deficient mice fed the HFD. Of note, this improvement occurred irrespective of the absence of DUSP2 having no impact on HFD-induced obesity, immune cell recruitment or inflammation in the WAT. The increased insulin sensitivity in HF-fed female dusp2−/− mice was modest, with these mice remaining markedly glucose intolerant and insulin resistant compared to mice fed the SCD. Irrespective of diet, we also observed that IL-10 gene expression was significantly lower in mice remaining markedly glucose intolerant and insulin resistant compared to mice fed the SCD. Of note, this difference in IL-10 expression is unlikely to be of physiological significance.

With various PTPs, such as PTP1B [29], currently the subject of intense drug discovery efforts in the context of obesity and insulin resistance, specific DUSP family members found to control MAPK dependent obesity, inflammation and insulin resistance may represent potential targets in the treatment of metabolic disease. Using mice that lack DUSP2, a nuclear phosphatase shown to promote the production of pro-inflammatory cytokines, nitric oxide and prostaglandin E2 by activated macrophages and mast cells [10], we assessed whether DUSP2 represented a therapeutic target by which MAPK-dependent inflammatory gene expression associated with obesity could be suppressed. Our data provide compelling evidence that DUSP2 does not play a non-redundant role in the development of obesity or obesity-associated inflammation and its related pathologies, despite these conditions being underpinned by a strong inflammatory component.

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Author Contributions
Conceived and designed the experiments: GIL MAF SG AB. Performed the experiments: GIL MJK HLK KLG EE. Analyzed the data: GIL MJK HLK EE. Contributed reagents/materials/analysis tools: RJG SG. Wrote the paper: GIL MJK HLK KLG AB MAF SG.

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