Myeloid-cell protein tyrosine phosphatase-1B deficiency in mice protects against high-fat diet and lipopolysaccharide induced inflammation, hyperinsulinemia and endotoxemia through an IL10 STAT3 dependent mechanism.

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Myeloid-PTP1B and inflammation

Abstract

Protein-tyrosine phosphatase-1B (PTP1B) negatively regulates insulin and leptin signalling, rendering it an attractive drug target for treatment of obesity-induced insulin resistance. However, some studies suggest caution when targeting macrophage-PTP1B, due to its potential anti-inflammatory role. We assessed the role of macrophage-PTP1B in inflammation and whole body metabolism using myeloid-cell (LysM)-PTP1B-knockout mice (LysM-PTP1B). LysM-PTP1B mice were protected against LPS-induced endotoxemia and hepatic damage, associated with decreased pro-inflammatory cytokine secretion *in vivo*. *In vitro*, LPS-treated LysM-PTP1B bone-marrow-derived-macrophages (BMDMs) displayed increased *IL10* mRNA expression, with a concomitant decrease in *TNFα* mRNA levels. These anti-inflammatory effects were associated with increased LPS- and IL10-induced STAT3 phosphorylation in LysM-PTP1B BMDMs. Chronic inflammation induced by high-fat (HF)-feeding led to equally beneficial effects of macrophage-PTP1B deficiency; LysM-PTP1B mice exhibited improved glucose- and insulin tolerance, protection against LPS-induced hyperinsulinemia, decreased macrophage infiltration into adipose tissue and decreased liver damage. HF-fed LysM-PTP1B mice had increased basal and LPS-induced IL10 levels, associated with elevated splenic STAT3 phosphorylation, *IL10* mRNA expression, and expansion of cells expressing myeloid markers. These increased IL10 levels negatively correlated with circulating insulin and ALT levels. Our studies implicate myeloid-PTP1B in negative regulation of STAT3/IL10-mediated signalling, highlighting its inhibition as a potential anti-inflammatory and anti-diabetic target in obesity.

Introduction

Protein tyrosine phosphatase-1B (PTP1B) is a non-receptor tyrosine phosphatase, identified as an attractive drug target for conditions associated with metabolic syndrome as well as potential anti-cancer therapeutic, due to its diverse involvement in regulation of various cell signalling cascades. Evidence to support the notion that PTP1B inhibition may be beneficial in states of over-nutrition and insulin resistance was demonstrated in mice with a global- (1, 2), as well as tissue-specific-PTP1B deletion (3-6).

Insulin resistance in adipose, muscle and liver is exacerbated in obese states, due to the underlying presence of chronic low-grade inflammation and macrophage infiltration into these tissues. White adipose tissue (WAT) from obese subjects contains greater numbers of infiltrating pro-inflammatory macrophages in comparison to lean counterparts, and these cells secrete cytokines such as tumour necrosis factor-alpha (TNFα), interleukin-1 beta (IL1β), and interleukin-6 (IL6), which can impair systemic insulin sensitivity (7-11). This process is enhanced further by the release of chemokines, such as monocyte-chemoattractant protein-1 (MCP1), which recruits more activated tissue-macrophages into WAT (12). Saturated fatty acids are able to directly induce the expression of these pro-inflammatory cytokines via activation of the NF-κB pathway, and the receptor primarily responsible for potentiating this effect has been identified as the macrophage toll-like receptor (TLR)4 (13). Mice with myeloid-TLR4 deletion are protected against high-fat (HF) diet-induced inflammation, adipose macrophage infiltration and insulin resistance (14).

It has been postulated that PTP1B may act as a negative regulator of TLR4 signalling in macrophages, since *in vitro* PTP1B knock-down in the Raw 264.7 macrophage cell line resulted in elevated production of TNFα, IL6 and IFNβ, following challenge with a variety of TLR ligands (15). PTP1B overexpression in the same cell line, caused a concomitant

decrease in pro-inflammatory cytokine production in response to LPS- and palmitate-challenge (15, 16). Similarly, splenic macrophages isolated from global PTP1B$^{-/-}$ mice were found to be highly sensitive to LPS-induced iNOS expression and nitric oxide production, in addition to elevated interferon gamma (IFNγ)-induced phosphorylation of STAT1 (17). In the same study, *in vivo* LPS-challenge increased sensitivity to endotoxic-shock in PTP1B$^{-/-}$ mice, associated with increased systemic production of interleukin-12 (IL12) and IFNγ. However, since these studies were performed in mice with global PTP1B deletion, and PTP1B regulation of cell function/signalling varies in a cell-specific manner (3-6), we set out to definitively establish the *in vivo* role of macrophage-PTP1B, by assessing the regulation of inflammation and whole body metabolism in LysM-PTP1B knockout mice.

Research Design and Methods

Animal studies. All animal procedures were performed under a project license approved by the UK Home Office under the Animals (Scientific Procedures) Act 1986 (PPL60/3951). $PTP1B^{fl/fl}$ and mice expressing Cre recombinase (Cre) under the control of the LysM-promoter have been described previously (3). To generate myeloid-$PTP1B^{-/-}$ mice, $PTP1B^{fl/fl}$ mice were crossed with LysM-Cre mice (18) and back-crossed 9 times to pure C57BL/6J mice. DNA extraction and genotyping were performed as described previously (3). Age-matched male mice were studied and compared to $PTP1B^{fl/fl}$ and LysM-Cre control littermates. Mice were group-housed and maintained at 22-24ºC on 12-hour light/dark cycle with free access to food/water. At weaning (21-days), mice were placed on standard 3.4% fat chow-pellet diet (Rat and Mouse Breeder and Grower, Special Diets Services, DBM, Scotland) or HFD (Adjusted Calories Diet, 55% fat, Harlan Teklad, USA) for 29 weeks and weight recorded weekly. The approximate fatty-acid profile of Adjusted Calories Diet (% total fat) was 28%-saturated, 30%-trans, 28%-monounsaturated (cis) and 14%-polyunsaturated (cis). For endotoxemia experiments, 50-week old chow-fed and HF-fed mice were fasted for 2-hours and injected intraperitoneally with LPS (1 mg/kg, 0.5 mg/kg respectively; Merck). Mice were observed for signs of sepsis, including reduced mobility, fur-ruffling, and conjunctivitis (19). 24- or 3-hours post-LPS injection (stated in figure legends), mice were sacrificed by cervical dislocation and tissues/blood harvested (3).

Blood analysis. Tail-blood glucose from fasted (5-hours) mice was measured using glucometers (Accu-Check, Burgess Hill, UK) (6). Serum TNFα, IL6, MCP1, insulin, leptin, PAI1, and resistin were measured using adipokine multiplex-kit (Millipore, UK) and serum IL10 levels determined by ELISA (R&D Systems, Minneapolis, USA). Alanine-transferase (ALT) (BioVision) and serum glucose (Thermo Scientific) levels were assayed per manufacturer's instructions. Glucose tolerance tests (GTT) and insulin tolerance tests (ITT)

were performed as described (2, 6). For hematocrit-level determination, whole-blood was collected in heparinised micro-hematocrit capillary tubes and centrifuged for 3-minutes. The percentage hematocrit value was determined by measuring the length of the red-blood cell layer and calculating as a percentage of the total height of the column sample.

Isolation of T-lymphocytes. Single-cell suspensions were made from spleens and lymph nodes by pressing through a 70-μm strainer (BD Falcon). After red-blood cell lysis (Sigma), cells were labelled with anti-L3T4 (CD4) microbeads (Milteny Biotec) and sorted using LS-columns (Milteny Biotec).

Isolation and stimulation of bone-marrow-derived-macrophages (BMDMs). BMDMs were obtained by flushing out femurs and tibiae with sterile PBS (Lonza), as described previously (6). Mature macrophages were seeded in 6-well tissue-culture plates (1 x 10^6 cells/well) and serum-starved for 16-hours prior to stimulation with 100 ng/ml LPS (InvivoGen), 10 ng/ml IL10 (Peprotech), or 20 ng/ml IL6 (Peprotech).

Cell line work Stable knock-down of PTP1B in RAW 264.7 cells (murine-macrophage cell line) was performed using short-hairpin RNA constructs as previously described (20)

Immunoblotting. Cells and tissues were lysed in radioimmunoprecipitation-assay (RIPA) buffer containing fresh sodium-orthovanadate and protease-inhibitors (21). Proteins were separated by 4-12% SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were performed using antibodies from Cell Signaling (NEB, Hitchin, UK) (unless stated otherwise) against pERK1/2 MAPK T202/Y204, pIKKα/β S176/S180, IκBα, p-c-jun S63, pP38 T180/Y182, pSTAT1 Y701, pSTAT3 Y705, STAT3, BCL-2, iNOS, SHP1, pAkt/PKB S473, pS6 ribosomal protein S235/236, pGSK 3α/β, pIR Y1162/1163 (Invitrogen), SHP2 (Santa Cruz), ERK2 (Santa Cruz), TC-PTP (R&D Systems), pJNK/SAPK

T183/Y185 (R&D Systems), β-actin (Sigma) and PTP1B (Millipore). Immunoblots were visualized using enhanced-chemiluminescence, and quantified by densitometry scanning using Bio1D-software (PeqLab, Fareham, UK).

Gene-expression analysis. Cells/tissues were homogenized in TriFast reagent (Peqlab, UK) (20). cDNA synthesis was carried out from 1 µg of RNA using Tetro cDNA-synthesis kit (Bioline). Quantitive real-time PCR was performed using Light-Cycler 480 (Roche) and gene expression of *iNOS, TNFα, IL6, MCP1, IL1β, IL1α, IL10* and *BCL2* determined relative to the most stable reference gene (*YWhaz*, *NoNo*, or *β-actin*) which was identified using a web-based reference gene assessment tool (http://www.leonxie.com/referencegene.php?type=reference). Primer sequences are provided in supplementary material.

Cytokine quantification. TNFα, IL6 and IL10 concentrations in BMDM supernatants were quantified by ELISAs (R&D Systems, Minneapolis, USA) or multiplex-ELISA (Millipore), according to the manufacturer's instructions.

Nitrite-determination. Nitric-oxide production by BMDMs was quantified by determining the concentration of nitrite present in supernatants using Griess-reaction (22).

Adipose-immunohistochemical staining. Ethanol-fixed, paraffin-embedded adipose tissue sections were stained as previously described (23) using rat anti-mouse F4/80 (AbD Serotec MCA497RT), CD68 (Abcam ab31630), and iNOS (Cell Signaling).

H&E staining. Liver and spleen tissue was fixed in formaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Flow cytometry. Single cell suspensions were made by pressing spleens through cell-strainers and cell-suspensions were centrifuged at 900*g* for 5-minutes. Red-blood cells were lysed for 1-minute with red-blood cell lysis-buffer (Sigma) and washed by centrifugation at 900*g* for 5-minutes in PBS without Ca^{2+} and Mg^{2+}, 5% fetal-bovine serum, and 5 mM EDTA.

1x 10^6 cells per label were blocked for 30-minutes in the dark with rat anti-mouse CD16/CD32 (BD, 0.5 µg/reaction) and then labelled with optimised quantities of antibodies (PerCP-Cy5.5 (BD 550954), CD8 V450 (BD 560469), CD3 AF488 (BD 557666), CD45 PE (BD 553089), CD11b AF488 (BD 557672), CD11c V450 (BD 560521), Gr-1 APC-Cy7 (BD 557661), MHC-II PE (BD 557000), Ly6C PerCP-Cy5.5 (BD 560602), Ly6G APC (BD 560595), F4/80-AF647 (AbD Serotech). Data was acquired on BD LSR II and analysed using DiVa-software.

Data analysis. Data are expressed as mean ± SEM. Statistical analyses were performed using correlation analyses, one-way ANOVA with Tukey's multiple comparison post-tests, two-way ANOVA with Bonferroni-multiple comparisons post-tests, and two-tailed Student's t-tests, as appropriate, using GraphPad Prism 5 statistical-software. Statistical significance is indicated as follows: (*) p-value < 0.05; (**) p-value < 0.01; and (***) p-value < 0.001.

Results

LysM-PTP1B mice are protected against LPS-induced endotoxemia

PTP1B deletion was achieved in BMDMs isolated from LysM-PTP1B mice, without affecting PTP1B levels in WAT, liver, muscle (*fig. 1A*) or $CD4^+$ T-lymphocytes (*fig. 1B*). A deletion efficiency of ~75% was determined in LysM-PTP1B BMDMs (n = 4) compared to control (n = 5) (*fig. 1C and D*). There were no body weight differences (*fig. 1E*) and glucose- (GTT) and insulin-tolerance (ITT) were unaltered between LysM-PTP1B and control mice on chow-diet (*fig 1F and IG, respectively*). Fed- and fasted-metabolic parameters were unchanged in the absence of myeloid-PTP1B (*table 1*). To establish whether macrophage-PTP1B plays a role in TLR4 signalling, LysM-PTP1B mice with confirmed PTP1B deletion (*fig 1C*) and littermate controls were injected with low-dose LPS (1 mg/kg) and their response to endotoxin monitored. Twenty-four hours post-LPS, control mice exhibited obvious signs of endotoxiemia, including closed eyes, shivering, reduced mobility and ruffled fur (*fig 1H and supplemental videos V1-2*), whereas LysM-PTP1B littermates were completely protected (*fig 1I and videos V3-4*). LysM-PTP1B mice had decreased serum TNFα and IL6 levels compared to controls, although this did not reach significance (TNFα: 0.04 ± 0.005 ng/ml vs 0.1 ± 0.03 ng/ml respectively, (P=0.18; 2-tail t-test)*;* IL6: 3.7 ± 0.9 ng/ml vs 14.3 ± 5.3 ng/ml, respectively (P=0.06; 2-tail t-test) (*table 2*). At 3-hours post-LPS, there was also a trend towards increased IL10 levels in LysM-PTP1B mice (0.89 ± 0.12 ng/ml vs. 0.67 ± 0.03 ng/ml, (P = 0.12; 2-tail t-test) although this was not observed at 24-hours post-LPS (*table 2).* Furthermore, levels of hepatic STAT1 and STAT3 phosphorylation were significantly lower in LysM-PTP1B mice, indicative of decreased LPS-induced hepatic-inflammation (*fig 1J and IK*).

LysM-PTP1B macrophages exhibit altered cytokine kinetics

To assess whether the anti-inflammatory phenotype observed in LPS-challenged LysM-PTP1B mice is due specifically to the absence of macrophage-PTP1B, isolated BMDMs were stimulated with 100 ng/ml LPS for varying durations, up to 24-hours *in vitro*.

Elevations in *IL10* mRNA expression in LysM-PTP1B BMDMs following LPS-stimulation were observed at both 4- and 24-hours (*fig. 2A*), which was associated with increased STAT3 mRNA expression 4-hours post-LPS treament (*fig 2B*). *TNFα* transcript levels were concomitantly down-regulated following 4-hours of LPS-treatment in LysM-PTP1B cells (*fig 2C*). *Supplementary table 1* displays additional measures of pro-inflammatory cytokines and markers of M1 and M2 macrophages.

In keeping with previously published data showing that isolated macrophages from global PTP1B$^{-/-}$ mice are highly sensitive to LPS-induced *iNOS* expression (17), we also found increased levels of LPS-stimulated *iNOS* mRNA transcript in the absence of macrophage-PTP1B (*fig 2D).* Supernatants harvested from LysM-PTP1B BMDMs challenged with LPS for 6-hours contained increased IL10 (*fig 2E*), and decreased TNFα levels (fig *2F*). Supernatant nitrite (*fig 2G*) and iNOS protein levels (*fig 2H and 2I*) were higher in LPS-stimulated LysM-PTP1B macrophages compared to respective control cells.

Macrophage-PTP1B regulates JAK/STAT signalling in LPS- and IL10-treated LysM-PTP1B macrophages and RAW 264.7 stable knockdown cells.

We found increased levels of phosphorylated-STAT1 alpha and beta isoforms in LysM-PTP1B macrophages treated with LPS for 24-hours (*fig 3A, 3C*), which is in agreement with previous reports and is a likely explanation for the elevated iNOS and nitrite

levels produced by these cells (17). The level of phosphorylated-STAT3 was also significantly increased in LysM-PTP1B cells following 24-hours of treatment with 100 ng/ml LPS (*fig 3B, 3C*). The ability of endogenous IL10 to directly induce STAT3 activation is a likely cause for the heightened levels of phosphorylated-STAT3 observed in PTP1B-deficient macrophages, since the levels of IL10 detected in respective supernatants were also elevated (24).

To assess this, LysM-PTP1B and control macrophages were stimulated with IL10 for varying durations and levels of STAT3-phosphorylation determined (*fig 3D*). IL6, which also activates STAT3, was used to allow for a direct comparison (*fig 3E).* An elevation in IL10-induced pSTAT3 was observed following 60- and 120-minute stimulation (*fig 3F*) in the absence of macrophage-PTP1B, which was not the case for IL6-induced STAT3-phosphorylation (data not shown). There was a negative correlation between PTP1B protein-levels present in each cell batch (*fig 3G*) and the quantity of phosphorylated-STAT3, following 120-minute IL10-treatment (*fig 3H*) suggesting a dose-dependent effect of STAT3-dephosphorylation by PTP1B. RAW 264.7 cells with PTP1B stable knockdown (*fig 3I*) also showed increased LPS-induced pSTAT1-Y701 (*fig 3J*), pSTAT3-Y705 and iNOS (*fig 3K*) and increased IL10-induced pSTAT3-Y705 (*fig 3L*).

Previous studies implicated macrophage-PTP1B in negative regulation of MAPK and NFκB signalling cascades, initiated by various TLR ligands (15). In our study, macrophage-PTP1B deficiency *in vitro* did not affect phosphorylation of c-Jun (component of MAPK signalling; *supplementary fig 1*) nor phosphorylation of p65 (data not shown) and associated degradation of IκBα (components of NFκB signaling; *supplementary fig 1),* following LPS-stimulation at various time-points. More detailed analysis of these pathways revealed no

alterations in the levels of phosphorylated ERK 1/2 (*supplementary figure 2*), p38, JNK 1/2, or IKKα/β (*supplementary fig 3*).

LysM-PTP1B mice show improved glucose homeostasis in a model of HF-diet (HFD) feeding, protection against LPS-induced hyperinsulinemia, decreased WAT inflammation and hepatic damage.

Since LysM-PTP1B mice were protected from an acute inflammatory challenge, we tested *in vivo* effects of myeloid-PTP1B deficiency under states of chronic low-grade inflammation, caused by long-term (29 weeks) HFD-feeding (23).

Despite no alterations in body weight (*fig 4A*), glucose tolerance tests (GTT) revealed improved ability to clear exogenous glucose in LysM-PTP1B HFD-fed mice (*fig 4B*). The area under the curve (AUC) also confirmed that LysM-PTP1B mice were more glucose tolerant than control mice (fig *4C*). LysM-PTP1B mice were also more insulin-tolerant with decreased blood glucose levels at 15, 90, and 120 min post-insulin injection (*fig 4D*), and decreased AUC during ITT (*fig 4E*).

Further, acute pro-inflammatory LPS-challenge led to hyperinsulinemia in HFD-fed control mice (75% increase from basal). Strikingly, LysM-PTP1B mice were completely protected against LPS-induced hyperinsulinemia (no increase from basal) (*fig 4F*) and post-LPS insulin levels were significantly lower in LysM-PTP1B compared to control mice. Post-LPS blood glucose levels were also lower in LysM-PTP1B mice (*fig 4G*). Additional measures of metabolic parameters and circulating cytokines are shown in *tables 1* and *2*, respectively.

HFD-fed LysM-PTP1B and control mice were injected with either saline or insulin (10mU/g body weight) and components of the insulin signalling pathway investigated in muscle, liver and WAT. There were no significant differences in insulin-stimulated insulin receptor phosphorylation between the two genotypes in muscle, (*fig 4H*), liver or WAT (Supplementary figures *S4* and *S5*, respectively),

F4/80 and CD68 immunohistochemical staining of WAT from HFD-fed LysM-PTP1B mice revealed a trend towards decreased expression of these markers when compared to controls (*fig 4I*). To confirm this quantitatively, we performed qRT-PCR analysis of WAT, which showed lower transcript levels of *F4/80* and *TNFα* and a trend for decreased expression of *CD68* (P = 0.09; 2-tail t-test) in LysM-PTP1B mice (*fig.* 4*J*), indicative of decreased macrophage infiltration into WAT and associated inflammation. HFD-fed LysM-PTP1B mice also had lower levels of hepatic lipid accumulation (*fig 4K and 4L*), which was associated with decreased serum ALT levels in these mice (*fig. 4M)*, indicative of improved liver function. Furthermore, LysM-PTP1B mice exhibited increased expression of the anti-apoptotic gene *BCL2* (*fig 4N*).

HFD-fed LysM-PTP1B mice have increased circulating IL10 levels, an elevation in spleen cells expressing myeloid markers, and increased levels of splenic phospho-STAT3.

Basal circulating levels of the anti-inflammatory cytokine IL10 were elevated in HFD-fed LysM-PTP1B compared to control mice (*fig 5A*). Acute LPS-challenge led to an induction of IL10 in both control and LysM-PTP1B mice, which led to a further 120% elevation in IL10 levels in LysM-PTP1B (*fig 5B*). Furthermore, we found a negative correlation between serum IL10 and ALT levels (*fig. 5C*), as well as serum IL10 and insulin levels (*fig 5D*) in HFD-mice following LPS-injection, suggesting that elevated circulating

IL10 levels are strongly associated with the anti-inflammatory role of myeloid-PTP1B deficiency.

Despite the strong anti-inflammatory phenotype of LysM-PTP1B mice, these mice (but not control mice) developed splenomegaly on HFD (*fig 5E)* with a mean spleen weight of 0.48 ± 0.05g (n = 14) compared to 0.14 ± 0.02g (n = 13) ($P \leq 0.0001$; 2-tailed T-test) which was also significant when corrected for body weight (*fig 5F)*. To assess if these mice were anaemic, hematocrit testing was performed on whole blood and no differences were found between LysM-PTP1B (36.6 ± 1.1 %; n = 9) and control mice (38.9 ± 1.0 %; n = 3). H&E staining of spleen sections revealed unremarkable histology (*fig. 5G*). Spleen size was found to strongly correlate with circulating IL10 levels under basal and LPS-stimulated states (*fig 5H* and *5I*). Splenic-IL10 mRNA levels correlated with circulating IL10 levels following LPS-treatment, suggesting that the elevation in systemic IL10 seen in HFD-fed LysM-PTP1B is, at least in part, spleen-derived (*fig 5J*).

Flow-cytometry analysis of splenocytes established that the percentage of cells expressing the granulocyte/monocyte marker Gr1, and the monocyte marker Ly6C, were significantly increased in LysM-PTP1B cells compared to controls and a trend for increased expression of Ly6G was noted (*P=0.09*) (*fig 5K*). There were no alterations in the percentage of cells expressing CD3, CD4, CD8, B220, CD11c, CD11b, F4/80 and MHCII; however, when normalised against total cell number, a significant increase in absolute cell numbers expressing all markers was established for LysM-PTP1B mice compared to controls (*supplementary table 2*).

Spleen lysates from HFD-fed LysM-PTP1B mice revealed an increase in the phosphorylation of STAT3α and STAT3β isoforms compared to control lysates (*fig L* and *5M*). Using antibodies against total STAT3, a significant increase in the alternatively spliced

STAT3β isoform was noted in LysM-PTP1B mice (*fig 5L* and *5M*). Interestingly, the splenic level of the anti-apoptotic protein BCL2, which is regulated at the gene level by STAT3, was also increased in these mice, which could contribute towards the splenomegaly phenotype observed (*fig 5L* and *5M*).

Discussion

Previous reports have implicated macrophage-PTP1B in the negative regulation of TLR4-mediated inflammatory signalling; however, these studies have been restricted to *in vitro* cell line analysis or characterization of responses in PTP1B global knock-out mice (15-17, 25). To definitively address the role of macrophage-PTP1B in inflammatory signalling, we have generated mice with myeloid-specific PTP1B deletion and interrogated their whole body physiology and signalling in isolated macrophages. These mice exhibited an LPS-tolerant phenotype *in vivo*, which was mirrored by the attenuation of pro-inflammatory cytokine expression in LPS-treated BMDMs lacking PTP1B *in vitro*. A likely explanation for the down-regulation of *TNFα* observed *in vitro* was the concomitant increase in transcription and secretion of the anti-inflammatory cytokine, IL10. This was further reinforced by heightened levels of phosphorylated-STAT3, which is known to mediate IL10-driven repression of inflammatory targets (26). A similar increase in phosphorylated-STAT3 was observed when macrophages lacking PTP1B were challenged with IL10, *in vitro*. The IL10R engage JAK1 and Tyk2 following receptor activation leading to the phosphorylation and activation of STAT3 (27, 28) and since Tyk2 is a known substrate of PTP1B (29), this is a plausible reason for the hyperphosphorylation of STAT3 observed in the absence of PTP1B. Furthermore, activated STAT3 has been shown to control the expression of the IL10 promoter, leading to a positive feedback mechanism (30). In summary, the altered cytokine profile displayed by these cells and their increased sensitivity to IL10 may be responsible, at least in part, for the state of LPS-tolerance observed in LysM-PTP1B mice 24-hours after receiving low-dose endotoxin *in vivo*.

Our *in vitro* analysis also revealed increased STAT1 phosphorylation, leading to increased iNOS and nitrite production in LPS-treated LysM-PTP1B BMDMs, which is in agreement with previous findings of LPS-treated spleen-macrophages from global knock-out

mice (17). Although heightened levels of NO have been partly implicated as a cause for increased endotoxin sensitivity in PTP1B$^{-/-}$ mice, no such phenotype was observed in LysM-PTP1B mice. These findings do however add to a large body of evidence that links PTP1B to the regulation of JAK-STAT activation (3, 29, 31-34).

The anti-inflammatory phenotype observed in endotoxin-challenged LysM-PTP1B mice was similarly replicated in our long-term-HFD-feeding study, which is known to chronically increase plasma-LPS concentration and has thus been termed metabolic endotoxemia (23). Most remarkably, *in vivo* LysM-PTP1B mice exhibited increased basal- and LPS-induced levels of circulating IL10 compared to control animals, which we found to negatively correlate with insulin and ALT levels. The insulin-sensitizing effect mediated by IL10 has been widely documented before. Low circulating levels of IL10 have been associated with obese and insulin-resistant states, as have polymorphisms and haplotypes of the IL10 promoter, and IL10 has been shown to mediate insulin-sensitizing effects in adipose, liver and skeletal muscle by suppressing the deleterious effects of TNFα and IL6 on insulin signalling (35-40). Furthermore, a shift towards an alternatively activated, anti-inflammatory M2-macrophage phenotype in adipose tissue has been associated with increased expression of IL10 (38).

The source of elevated IL-10 in HFD-fed LysM-PTP1B mice suggests it to be splenic in origin, due to the close correlations noted between circulating IL10, spleen size and splenic *IL10* mRNA expression. Further analysis of the spleens from these mice revealed an increased expression of the anti-apoptotic protein BCL2, which is regulated by STAT3 and may partly explain the splenomegaly phenotype (41) and an elevation in the alternatively spliced STAT3β isoform. Isoform-specific knock-out models have demonstrated that STAT3β plays a crucial role in inhibiting the acute-inflammatory responses, whereby mice

lacking STAT3β showed increased susceptibility and impaired recovery following *in vivo* challenge with LPS (42).

Increased phosphorylation of splenic STAT3 found in HFD-fed LysM-PTP1B mice, is in agreement with our *in-vitro* BMDM studies, as well as a recent study confirming STAT3 as a substrate of myeloid-PTP1B (34), which demonstrated that global-PTP1B-/- mice are protected against experimental colitis due to a STAT3/JAK2-mediated expansion of myeloid-derived suppressor cells (MDSCs) (34). Our data also provides evidence that this mechanism is, at least in part, applicable to the anti-inflammatory phenotype observed in HFD-fed LysM-PTP1B mice. States of chronic inflammation, which could be extended to encompass metabolic endotoxemia, are known to expand immunosuppressive MDSC populations (CD11b+ Gr1+ cells) via elevations in factors including pro-inflammatory cytokines, prostanoids and growth factors that lead to persistent STAT3 activation (43). In the absence of myeloid-PTP1B we observe increased splenic phospho-STAT3, elevated numbers of splenic cells expressing myeloid markers and marked splenomegaly, which would be indicative of MDSC expansion (43). In addition to the ability of MDSCs to inhibit T-cell activation (44, 45) and NK-cell tumour cytotoxicity (46), these cells are known to interact with macrophages by secreting elevated levels of IL-10, which promote macrophage polarization towards the M2 macrophage phenotype (47). It is therefore plausible to postulate that in the absence of myeloid-PTP1B, chronic HFD-feeding leads to a STAT3-dependent expansion of IL-10-secreting splenic MDSCs and possibly M2 macrophages, which alleviate hepatic and adipose inflammation and enhance insulin sensitivity. *Figure 6* displays a schematic depicting the postulated mechanism responsible for the beneficial effects observed for HFD-fed LysM-PTP1B mice.

In summary, this study provides evidence that LysM-PTP1B mice exhibit improved glucose tolerance and suppressed inflammatory responses in HFD-fed and endotoxemic

mouse models. This is contrary to previous reports that have assigned an anti-inflammatory function to macrophage-PTP1B, and will therefore help allay concerns relating to the application of PTP1B inhibitors in the clinical setting and open up new avenues for the use of PTP1B inhibitors as anti-inflammatory agents.

Author Contributions

LG and KS designed and performed experiments. AC, EL, CO, AA, CMG, JW performed experiments. JF and HW suggested experiments and reviewed the manuscript. LG, NM and MD conceived and designed the experiments and wrote the manuscript.

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Dr. Mirela Delibegovic is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. An abstract on this work was presented at the Diabetes UK Professional Conference 2013 and selected as an oral presentation.

There is no potential conflict of interest relevant to this article.

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Table 1Fed and fasted metabolic parameters (chow)

CHOW DIET					
Analyte	**Genotype**	**Fed levels**	**Signficance level**	**Fasted levels**	**Significance level**
Glucose (mg/dL)	Control	165.21 ± 10.79	*P = 0.14*	112.87 ± 10.16	*P = 0.69*
	LysM PTP1B	143.49 ± 4.87		106.89 ± 9.87	
Insulin (ng/ml)	Control	3.09 ± 0.20	*P = 0.93*	0.67 ± 0.12	*P = 0.59*
	LysM PTP1B	3.16 ± 0.90		0.76 ± 0.11	
Leptin (ng/ml)	Control	13.25 ± 1.41	*P = 0.09*	5.44 ± 0.99	*P = 0.11*
	LysM PTP1B	17.33 ± 1.43		8.40 ± 1.29	
Free fatty acids (mmol/l)	Control	1.51 ± 0.11	*P = 0.28*	2.55 ± 0.13	*P = 0.37*
	LysM PTP1B	1.35 ± 0.07		2.78 ± 0.23	
HF DIET					
Glucose (mg/dL)	Control	172.6 ± 5.39	*P = 0.86*	177.0 ± 12.37	*P = 0.33*
	LysM PTP1B	174.5 ± 9.83		197.0 ± 5.60	
Insulin (ng/ml)	Control	3.92 ± 0.38	*P = 0.25*	1.32 ± 0.22	*P = 0.71*
	LysM PTP1B	3.14 ± 0.44		1.17 ± 0.25	
Leptin (ng/ml)	Control	23.84 ± 1.85	*P = 0.69*	14.38 ± 1.67	*P = 0.56*
	LysM PTP1B	25.28 ± 3.37		16.60 ± 4.27	
Free fatty acids (mmol/l)	Control	1.54 ± 0.05	*P = 0.86*	1.65 ± 0.04	*P = 0.46*
	LysM PTP1B	1.52 ± 0.13		2.02 ± 0.80	

Data from chow-fed control (*n*=5) or LysM-PTP1B (*n*=4) mice and HFD-fed control (*n*=12) and LysM-PTP1B (*n*=6) mice are represented as mean ± SEM. Data were analyzed using two-tailed Student's t test (**P* < 0.05).

Table 2 Analysis of serum obtained from control and LysM PTP1B 3 hours and 24 hours following 1 mg/kg LPS injection.

CHOW					
Analyte (ng/ml)	**Genotype**	**3hr post-LPS**	**Significance level**	**24hr post-LPS**	**Significance level**
TNF-α	Control	0.38 ± 0.03	*P = 0.38*	0.10 ± 0.03	*P =0.18*
	LysM PTP1B	0.47 ± 0.07		0.04 ± 0.005	
IL-6	Control	149.0 ± 10.31	*P = 0.25*	14.3 ± 5.3	*P = 0.09*
	LysM PTP1B	134.4 ± 12.30		3.7 ± 0.9	
IL-10	Control	0.67 ± 0.03	*P = 0.12*	0.73 ± 0.13	*P = 0.7*
	LysM PTP1B	0.89 ± 0.12		0.65 ±0.07	
MCP-1	Control	*ND*		3.46 ± 1.60	*P = 0.3*
	LysM PTP1B	*ND*		1.54 ± 1.80	
Insulin	Control	*ND*		3.30 ± 0.96	*P = 0.5*
	LysM PTP1B	*ND*		2.50 ± 0.36	
Resistin	Control	*ND*		7.54 ± 1.87	*P = 0.5*
	LysM PTP1B	*ND*		6.00 ± 0.84	
PAI-1	Control	*ND*		7.41 ± 0.65	*P = 0.08*
	LysM PTP1B			11.71 ± 1.90	
HF DIET					
Analyte (ng/ml)	**Genotype**	**Basal**	**Significance level**	**3hr post-LPS**	**Significance level**
TNF-α	Control	*ND*		1.44 ± 0.28	*P = 0.2*
	LysM PTP1B	*ND*		2.10 ± 0.30	
IL-6	Control	0.03 ± 0.004	*P = 0.07*	60.78 ± 4.45	*P = 0.5*
	LysM PTP1B	0.11 ± 0.07		54.98 ± 10.41	
MCP-1	Control	0.06 ± 0.006	*P = 0.1*	55.99 ± 25.43	*P = 0.5*
	LysM PTP1B	0.04 ± 0.01		31.46 ± 14.46	
PAI-1	Control	5.00 ± 1.14	*P = 0.3*	5.15 ± 0.43	*P = 0.09*
	LysM PTP1B	6.88 ± 1.06		7.84 ± 1.97	

Data from 3 hour (n = 6 per genotype) and 24 hour (control mice n= 5, LysM PTP1B n= 4) LPS-injected mice. Multiplex analysis was used for 24 hour time-points (TNF, IL-6, Insulin, Resistin, PAI-1) and ELISAs used for the remaining time-points/analytes. *ND = not determined or too low to detect. Data are represented as mean ± SEM and analyzed using two-tailed Student's t test (*P < 0.05).

Figure legends

FIGURE 1. **LysM-PTP1B mice have unaltered glucose homeostasis but display an anti-inflammatory phenotype following *in vivo* challenge with low-dose lipopolysaccharide when compared to control littermates. (***A)*** Immunoblot depicting macrophage-specific deletion of PTP1B in LysM-PTP1B mice fed chow diet compared to littermate controls. Tissues shown are: (left to right) bone marrow-derived macrophages (BMDM), white adipose tissue (WAT), liver, and muscle. Blot was reprobed for SHP2, SHP1, and TC-PTP in addition to β-actin as a loading control to confirm specificity of PTP1B deletion. (***B)*** PTP1B levels were maintained in CD4+ T-lymphocytes harvested from LysM PTP1B mice and were not different to control cells. (***C)*** Immunoblot displaying PTP1B, SHP2 and β-actin protein levels in BMDM isolated from control mice (n = 5) compared to LysM-PTP1B (n = 4). (*D*) Corresponding quantification data of PTP1B protein levels relative to β-actin demonstrating a PTP1B deletion efficiency of 77 % in LysM-PTP1B BMDM compared to control levels (*E)* Mean body weight of male mice that had been back-crossed to C57BL/6J background for 9 generations, was unchanged when LysM-PTP1B (n = 6) were compared to control littermates fed standard 3.4% fat chow pellet diet for 10-weeks after weaning (n = 6). (*F)* Glucose tolerance testing (GTT) revealed no differences in the ability of LysM-PTP1B to clear glucose when compared to control mice and (*G*) insulin tolerance testing (ITT) demonstrated no differences between genotypes. 48-week old control mice (n = 5) and LysM-PTP1B littermates (n = 4) were injected with 1 mg/kg LPS *i.p*. Photographs showing an example of a (*H*) a control mouse compared to (*I*) a LysM-PTP1B mouse 24 hours after receiving 1 mg/kg LPS. Control mice exhibited more severe signs of endotoxic shock compared to mutant mice which were in better health. *(J)* Phosphorylation levels of STAT1 (Y701) and STAT3 (Y705) in liver tissue harvested following LPS injection were lower in LysM PTP1B mice compared to control (*K*). Control = white bars or open circles; LysM-PTP1B = black bars or closed circles). Data are expressed as mean ± SEM.

FIGURE 2. **Bone-marrow-derived macrophages (BMDM) isolated from LysM-PTP1B have increased IL-10 and iNOS and decreased TNFα gene expression and protein in response to LPS.** Treatment of BMDMs isolated from LysM-PTP1B mice (n = 4) and control mice (n = 4) with 100 ng/ml LPS for 4 hours and 24 hours revealed an increase in *IL-10* mRNA levels (P≤0.05; one-tailed T-test) (*A*) and STAT3 mRNA levels (4 hours post-LPS P≤0.01; two-tailed T-test) (*B*) in the absence of PTP1B. (*C)* Levels of pro-inflammatory *TNFα* transcripts were down-regulated in LysM-PTP1B cells compared to control cells following 4 hours challenge with LPS (P≤0.05; two-tailed T-test). *(D)* iNOS mRNA expression was elevated in LysM-PTP1B BMDMs following 4 hours LPS challenge (P≤0.05, respectively; two-tailed T-test). Expression levels were calculated relative to the most stable reference genes (*β-actin, YWhaz*, or *NoNo*). (*E*) Supernatants harvested from LysM-PTP1B macrophages following 6 hours treatment with 100 ng/ml LPS contained higher levels of IL-10 and (*F*) lower levels of TNF-α compared to control supernatants (P≤0.05; two-tailed T-test). *(G)* Nitrite levels were increased in supernatants harvested from LysM-PTP1B BMDMs stimulated for 24 hours with 100 ng/ml LPS compared to respective controls (P≤0.05; two-tailed T-test). (*H*). Quantification of immunoblot (*I*) showing elevated iNOS protein levels in LysM-PTP1B compared to control BMDM cells following challenge with 100 ng/ml LPS for 24 hours (P≤0.05; two-tailed T-test). Control = white bars; LysM-PTP1B = black bars. Data are expressed as mean ± SEM.

FIGURE 3**. LysM-PTP1B bone marrow-derived macrophages and a RAW 264.7 macrophage cell line with PTP1B knock-down display increased LPS-induced phosphorylation of STAT1 and STAT3 and increased IL10-induced phosphorylated STAT3, *in vitro* when compared to control cells.** *(A)* Immunoblot showing levels of phospho-STAT1 (Y701) and (*B*) phospho-STAT3 (Y705) in control and LysM-PTP1B BMDMs stimulated with 100ng/ml LPS for 24 hours. *(C)* Levels of phospho-STAT1 alpha ($P \leq 0.01$; two-tailed T-test) and beta ($P \leq 0.01$; two-tailed T-test), and phospho-STAT3 ($P \leq 0.05$; two-tailed T-test) were elevated in LPS-treated LysM-PTP1B BMDMs compared to control cells (phospho-STAT proteins are expressed relative to relevant total STAT protein). Immunoblot showing levels of phospho-STAT3 protein in LysM-PTP1B and control BMDMs induced by (*D*) 10 ng/ml IL10 and (*E*) 20 ng/ml IL6 for 30, 60, and 120 min. (*F*) Increased IL-10 phospho-STAT3 relative to total STAT3 in LysM-PTP1B BMDMs compared to control cells following 60 min ($P \leq 0.05$; two-tailed T-test) and 120 min ($P \leq 0.01$; two-tailed T-test) stimulation (*G*) Immunoblot displaying levels of PTP1B protein present in control and LysM-PTP1B BMDMs used for IL10 and IL6 stimulations and (*H*) the correlation between PTP1B protein levels and levels of phospho-STAT3 following l20 min IL-10-stimulation ($R^2 = -0.51$; $P \leq 0.01$) (*I*) Immunoblot showing effective PTP1B knock-down in RAW 264.7 cells transfected with PTP1B shRNA compared to cells transfected with empty vector only. (*J*) Increased levels of LPS-induced phospho-STAT1 (Tyr701), (*K*) phospho-STAT3 (Tyr705), and iNOS protein were observed for shPTP1B RAW 264.7 cells compared with vector only cells. (*L*) Levels of phospho-STAT3 (Tyr705) were also elevated in shPTP1B RAW 264.7 cells treated with IL10 during a time-course experiment (30, 60, and 120 min) when compared to levels in vector only RAW 264.7 cells. Control = white bars; LysM-PTP1B = black bars. Data are expressed as mean ± SEM.

FIGURE 4. **LysM-PTP1B mice fed high-fat (HF) diet are more glucose and insulin tolerant than littermate controls despite no alteration in body weight, are protected against LPS-induced hyperinsulinemia, have decreased levels of adipose tissue inflammation and lower levels of HF-induced liver damage.** *(A)* Body weights for LysM-PTP1B mice (n = 5) compared to control mice (n = 6) during an adjusted calorie diet containing 55% fat (HF-diet). *(B)* Following long-term HF dietary feeding, LysM-PTP1B mice (n = 5) were more glucose tolerant than controls (n = 6) 60 min post-glucose injection as determined by GTT ($P \leq 0.05$; two-tailed T-test). *(C)* GTT results expressed as area under the curve (AUC) showed significant improvement in glucose tolerance in the absence of PTP1B ($P \leq 0.05$; two-tailed T-test). (*D*) ITT of HF-fed LysM-PTP1B mice (n = 8) revealed increased insulin tolerance compared to HF-fed control mice (n = 6) at 15, 90, and 120 min post-insulin (1.1 mU insulin/g body weight) infusion (2-way ANOVA). *(E)* AUC for ITT was lower for LysM-PTP1B mice compared to controls ($P \leq 0.01$; two-tailed T-test). *(F)* Three hours after administration of 0.5 mg/kg LPS, circulating insulin levels measured in HF-diet fed control mice (n = 6) were significantly greater than basal levels ($P \leq 0.05$; two-tailed T-test) but this was not observed for LysM-PTP1B littermates (n = 5). LysM-PTP1B mice had significantly lower circulating insulin ($P \leq 0.01$; two-tailed T-test) and, *(G)* glucose ($P \leq 0.05$; two-tailed T-test) than control mice following LPS injection. *(H)* Muscle tissue immunoblot of insulin signalling pathway components after saline or insulin (10mU/g body weight) injection of control or LysM-PTP1B mice. *(I)* Representative images of control and LysM-PTP1B adipose tissue stained with F4/80 and CD68 and corresponding quantification data. *(J)* RT-PCR analysis of HF-fed mice revealed lower mRNA expression of *F4/80* and *TNF-α*, in adipose tissue extracted from LysM-PTP1B animals compared to control ($P \leq 0.05$; two-tailed T-test) and a trend for decreased *CD68* expression ($P = 0.09$; two-tailed T-test). *(K)*

Liver triglycerides were significantly lower in LysM-PTP1B tissue compared to control ($P \leq 0.001$; two-tailed T-test). *(L)* Hematoxylin and eosin staining of liver sections show a decreased presence of lipid accumulation in hepatocytes of LysM-PTP1B mice (lower panel) compared to control livers (upper panel). *(M)* Levels of serum alanine aminotransferase (ALT) were found to be lower in HF-fed LysM-PTP1B mice compared to controls ($P \leq 0.05$; two-tailed T-test). *(N)* Hepatic expression of anti-apoptotic mRNA *BCL-2* was significantly up-regulated in HF-fed, LPS-treated LysM-PTP1B mice compared to controls ($P \leq 0.01$; two-tailed T-test). Control = white bars or open circles; LysM-PTP1B = black bars or closed circles). Data are expressed as mean ± SEM.

FIGURE 5. **HF-fed LysM-PTP1B mice have increased circulating IL10 likely to originate from enlarged spleens containing elevated myeloid cell numbers and increased phospho-STAT3, *in vivo*.** *(A)* Levels of IL10 were increased in LysM-PTP1B mice (n= 4) following long-term HF-feeding compared to controls (n=9) (0.13 ± 0.04 and 0.03 ± 0.006 ng/ml, respectively; $P \leq 0.05$; two-tailed T-test) and *(B)* 3 hours after receiving an *i.p*. dose of 0.5 mg/kg LPS (3.18 ± 0.33 and 1.45 ± 0.17 ng/ml, respectively; $P \leq 0.001$; two-tailed T-test). *(C)* Serum IL10 levels were found to negatively correlate with ALT activity ($R^2 = 0.38$; $P < 0.05$) and *(D)* circulating insulin ($R^2 = 0.43$; $P < 0.05$) levels. *(E)* Photographs showing spleens harvested from HF-fed LysM-PTP1B mice compared to those from control mice on fl/fl and LysM-Cre backgrounds. *(F)* Increased spleen to body weight ratio for HF-fed LysM-PTP1B mice compared to controls ($P \leq 0.01$; two-tailed T-test). (*G*) Hematoxylin and eosin staining of spleen sections from representative control and LysM-PTP1B mice following HF-feeding. Spleen weight was found to correlate with circulating IL10 levels in *(H)* pre-LPS ($R^2 = 0.83$; $P \leq 0.01$) and *(I)* post-LPS ($R^2 = 0.76$; $P \leq 0.001$) states. *(J)* Circulating levels of IL10 were found to also correlate with levels of splenic IL10 mRNA ($R^2 = 0.43$; $P \leq 0.05$). (*K*) Flow cytometry analysis of spleen cells revealed an increase in the percentage of cells expressing the monocyte/granulocyte marker Gr-1 ($P \leq 0.05$; two-tailed T-test), the monocyte marker Ly-6C ($P \leq 0.05$; two-tailed T-test) and granulocyte marker Ly6G ($P = 0.09$). *(L)*. Immunoblot displaying increased levels of phospho-STAT3α relative to STAT3α ($P \leq 0.01$; two-tailed T-test), phospho-STAT3β relative to STAT3β ($P \leq 0.05$; two-tailed T-test), STAT3β and BCL-2 relative to β-actin ($P \leq 0.01$; two-tailed T-test) and BCL-2 ($P \leq 0.01$; two-tailed T-test) in spleens harvested from HF-fed LysM-PTP1B (n = 4) compared to control mice (n = 4) as quantified by relative densitometry (*M*).Control = white bars; LysM-PTP1B = black bars. Data are expressed as mean ± SEM.

FIGURE 6. **Schematic showing postulated mechanism responsible for the amelioration of hepatic and adipose inflammation leading to improved insulin sensitivity in HF-fed LysM-PTP1B mice.**