Title page

Title:

"A role for PTP1B in dendritic cell maturation, migration and T cell activation"

Short title: PTP1B regulation of dendritic cell function

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Authorship

C.M.G. conceived, designed, and performed experiments, analysed the data and wrote the manuscript. A.R.P. conceived, designed and performed experiments and analysed data. S.LS., I.P.K., T.Y. performed experiments and analysed data. E.M., L.G. performed experiments. C.V.G. analysed data. M.D. reviewed the manuscript. J.V.F suggested experiments, interpreted data and wrote the manuscript.

Abstract

Dendritic cells (DCs) are the major antigen-presenting cells bridging innate and adaptive immune responses, a function they perform by converting a quiescent DC to an active, mature DC able to activate naïve T cells. Here, we demonstrate that myeloid-cell specific genetic deletion of PTP1B (LysM PTP1B) leads to defects in lipopolysaccharide-driven bone marrow-derived DC (BMDC) activation associated with increased levels of phosphorylated Stat3. Mature DCs migrate and home to secondary lymphoid tissues to activate T cells. We demonstrate that myeloid-cell PTP1B deletion causes decreased migratory capacity of epidermal DC and reduced mature BMDC expression of the lymph node homing chemokine receptor CCR7 and BMDC chemotaxis to CCL19. Further, immature LysM PTP1B BMDCs display fewer podosomes, increased levels of phosphorylated Src at tyrosine 527 and loss of Src localisation to podosome puncta. In addition, we show decreased numbers of contacts and contact duration in BMDC:T cell co-cultures from LysM PTP1B mice. Finally, we show that LysM PTP1B BMDCs fail to present antigen to T cells as efficiently as control BMDCs. In summary, we provide first evidence for a key regulatory role for PTP1B in mediating a central DC function of initiating adaptive immune responses in response to innate immune cell activation.

Introduction

DCs are the most potent professional antigen-presenting cells (APC) connecting the innate immune response with the initiation of adaptive immunity (Steinman et al., 2003; Steinman, 2012). DCs are generated primarily in the bone marrow whence they enter the blood stream and populate the peripheral tissues where their default function is to promote tolerance (Steinman et al., 2003; Alvarez et al., 2008). In their immature state, DCs reside in peripheral tissues and traffic to the secondary lymphoid organs (SLO) in a slow "trickle" to promote immunological homeostasis by regulating responses to self antigens. Tissue-resident DCs respond to microbial challenge particularly in barrier tissues such as the skin and intestine where they are specialized in uptake, processing, and presentation of foreign antigens to T cells. Antigen processing induces a program of DC maturation characterised by upregulation of the expression of chemokine receptors particularly CCR7, co-stimulatory molecules (CD80, CD86, CD40) and MHC class I and II molecules on their surface. This process facilitates the migration of DCs from the peripheral tissues via lymph and blood to SLO (Alvarez et al., 2008; Randolph et al., 2008), and the presentation of antigen to naïve T cells. The ability of DCs to migrate in and out of these various compartments is key to their functions.

During migration, the DC navigates through the tissues to enter the afferent lymphatics. In this process it must release its relatively firm adhesive attachment to tissue cells (Van den Bossche et al., 2012) and invade the extracellular matrix. This is achieved by formation of protrusive/adhesive plasma membrane structures (podosomes) a general cellular phenomenon used by migrating immune cells to traverse dense tissues such as vascular basement membranes (Carman et al., 2007; Gawden-Bone et al., 2010) and is critical for immune cell function. For instance, lymphocytes use Src kinase-dependent podosomes and extended "invasive podosomes" to sense the surface of the endothelium and ultimately form transcellular pores (Carman et al., 2007). *In vitro* studies have shown that podosomes are small cylindrical structures containing a central branched-actin bundle core and an adhesive surrounding ring. They are initially formed as adhesive points of contact ("puncta"), partially comprised of trans-membrane αβ integrin - cytoskeletal connections, involving "inside-out signalling" *via* Src- containing intracellular protein complexes (Hoshino et al., 2013).

PTP1B (protein tyrosine phosphatase 1B) is a non-receptor tyrosine phosphatase that has multiple substrates, which, in turn, are involved in a wide range of fundamental cellular processes (Mertins et al., 2008). PTP1B is an attractive drug target for cancer and diabetes due to its regulatory role in cell and metabolic signalling (Elchebly et al., 1999; Bence et al., 2006; Delibegovic et al., 2009; Grant et al., 2014). PTP1B deficient mice are protected against the effects of high-fat diet, age-induced obesity and insulin resistance associated with chronic inflammation in white adipose tissue (Elchebly et al., 1999; Gonzalez-Rodriguez et al., 2012). Moreover, we recently demonstrated that myeloid-cell specific PTP1B knockout mice were protected against LPS-induced endotoxaemia and liver damage, which correlated with low levels of proinflammatory cytokine secretion *in vivo* (Grant et al., 2014). Furthermore, p38 mitogen-activated protein kinase is a substrate of PTP1B in B cell and loss of PTP1B in B cells is associated with autoimmunity in mice and humans (Medgyesi et al., 2014). Despite the increasing number of reports exploring the roles of PTP1B in immune cells, the role of PTP1B in dendritic cells is unknown.

Here we demonstrate that genetic deletion of PTP1B in LysM PTP1B mice leads to decreased migratory capacity of epidermal DCs from split skin explants, as well as a marked reduction in the ability of mature BMDCs to respond to the lymph node homing chemokine CCL19 *in vitro*. We also show that LysM PTP1B immature BMDCs display decreased ability to develop podosomes while simultaneously forming increased numbers of focal contacts. Interestingly, these phenotypes are consistent with increased levels of phosphorylated Src at Tyr527, which renders Src inactive and leads to loss of Src localisation to podosome puncta. Furthermore, we show decreased numbers and duration of BMDC:T cell contacts in co-cultures from LysM PTP1B mice, compared with co-cultures of control mice. Notably, these defects caused by loss of PTP1B in BMDCs correlate with lowered expression of molecules which contribute to the immunological synapse. Finally, we demonstrate that LysM PTP1B DC exhibit reduced ability to present antigen to T cells. In summary, we provide first evidence for a key regulatory role of PTP1B in DC physiology specifically in the process of DC maturation, a process central to the initiation of adaptive immunity. Hence, our studies highlight the potential of DC-specific targeting of PTP1B for intervention in regulation of the immune response.

Results

PTP1B depletion in BMDCs causes defects in LPS-driven DC maturation associated with increased phosphorylation of Stat3.

To study the function of PTP1B in DCs we used myeloid-specific PTP1B$^{-/-}$ mice (Grant et al., 2014). Differentiation of bone marrow progenitors cultured in GMCSF (Inaba et al., 1992) was determined by typical DC clustering, morphology and expression of double positive CD11c^{+}/CD11b^{+} cells on day 7 of culture. Cell differentiation was not affected by depletion of PTP1B (LysM PTP1B BMDCs 76% ±9 vs 81%±10 control BMDCs; p=0.3). PTP1B loss in BMDCs was confirmed by Western blotting (Fig. 1A). Maturation of DCs by 24 h treatment of BMDCs with LPS (Lau et al., 2008) caused no change in levels or localisation of PTP1B (Fig. 1A and B). We found no differences in the number of immature BMDCs between control and LysM PTP1B mice when DCs were generated *in vitro* from bone marrow precursors; however, we found a trend for increased iBMDC in spleens of unchallenged LysM PTP1B mice, compared to control unchallenged mice (Supplementary figure 1, panel A). In agreement with this finding T_{reg} cell number was increased in spleens of LysM PTP1B mice compared to the controls (Supplementary figure 1, panel B). In line with Zhang et al., 2013, wefound that the increase in immature cells in spleens of LysM PTP1B mice became significant when mice where challenged with high fat diet (Grant et al 2014).

Previous studies have shown that overexpression / hyper-activation of STAT3 causes impaired DC activation in addition to accumulation of immature DCs whereas inhibition of Jak2/STAT3 signalling results in strong activation of immature DCs (Nefedova et al., 2004; Nefedova et al., 2005). Hence, we investigated whether LysM PTP1B BMDCs display increased phosphorylation of Stat3 and if this affects their ability to become activated /mature in the presence of LPS. Control BMDCs display basal phosphorylation of Stat3 which disappears after 1 h LPS-treatment to increase again over basal levels in their fully mature state (Fig. 1 C). LysM PTP1B BMDCs constitutively display hyper-phosphorylation which is maintained after 1 h LPS-treatment and increases further after 24 h. We hypothesized that constitutively activated Stat3 phosphorylation may prevent normal DC maturation in LysM PTP1B BMDCs. We therefore examined the expression of cell surface markers of maturation by flow cytometry (Fig. 1D). We found that control BMDCs expressed low to intermediate levels of MHC II (Fig. 1D). As expected, treatment of control BMDCs

(MHC II low population) with LPS resulted in approximately a 5-fold increase in MHC II expression (shift from MHC II low towards MHC II high population; $p<0.0001$) compared to cells not exposed to LPS (Fig. 1D). 24 h LPS treatment also increased the expression of CD86 and CD40 by a factor of 65 ($p<0.0001$) and 9-fold ($p<0.0001$), respectively (Fig. 1D). However, LPS-treated PTP1B-deficient BMDCs displayed a 45% ($p=0.0005$), 25% ($p=0.01$) and 18% ($p=0.06$) lower expression of MHC class II, CD86 and CD40, respectively, compared to control LPS-treated BMDCs (Fig. 1D). The lower sensitivity to LPS was not due to decreased activation of TLR4 signalling, as shown by the lack of differences in phosphorylation of proteins downstream of TLR4 signalling or cytokine production (Supplementary figures 2 and 3).

Loss of PTP1B results in compromised migration of resident DC from skin explants and decreased chemotaxis of BMDC towards CCL19.

We first tested the ability of resident skin DCs (Langerhans cells) to migrate from skin explants *ex vivo* using cultured explants from mouse ear skin. For this, the migration of DC from split skin explants was evaluated histologically by determining the number of MHC Class II positive staining cells per unit area of explants after 3 days of culture *in vitro* and comparing this number with the number of cells per unit area in uncultured explants processed in parallel. The number of MHC-class II^+ DCs emigrating spontaneously from the skin epithelium was significantly decreased in LysM PTP1B mice i.e. more cells failed to migrate from LysM PTP1B epithelial sheets than in the controls (mean of 30.8 LysM PTP1B versus 23.8 control per microscopic field) (Fig. 2A). We next assessed *in vitro* chemotaxis of immature BMDCs and LPS-matured BMDCs using the chemokines CCL3 and CCL19 (Fig. 2B). Chemotaxis was performed in a transwell assay, using collagen-coated inserts. CCL3 is a pro-inflammatory chemokine which induces motility and migratory activity in a wide range of cells including immature but not mature DCs (as confirmed in Fig. 2B, left panel), while the action of CCL19 is restricted to mature cells expressing CCR7 which home to the SLO (as confirmed in Fig. 2B, right panel). Remarkably, deletion of PTP1B in immature BMDCs had no significant effect on their chemotactic response to the pro-inflammatory chemokine CCL3, while deletion of PTP1B in mature BMDCs decreased their responses to the homing chemokine CCL19 at of the

level of significance (p=0.05) (control =1.9±0.2 fold; LysM PTP1B =1.4±0.06) (Fig. 2B).

Maturation signals in DCs are known to trigger up-regulation of CCR7 (Alvarez et al., 2008). We investigated whether the chemotactic defects of LysM PTP1B BMDCs were due to differences CCR7 expression (Fig. 2C). Mature LysM PTP1B BMDCs cells exhibited both decreased surface (15% less CCR7 expression; p=0.0001) and intracellular (11.3% less CCR7 expression; p=0.01) levels compared to mature control BMDCs (Fig. 2C), suggesting that PTP1B was implicated in signalling processes involved in CCR7 synthesis and not in CCR7 trafficking.

Further, we found that the decrease in migration observed in LysM PTP1B mature BMDCs was associated with a significant increase in adhesion to a collagen substrate ($p < 0.05$) (Fig. 3) and was reflected in a decrease in the speed of random migration in these cells as observed after seeding cells on collagen coated plastic and assessing the speed of random migration using an automated cell tracking algorithm (Supplementary figure 4).

Collectively, these data indicate that decreased expression of CCR7 combined with a strong cell adhesion to collagen underlie the chemotactic defects towards CCL19 and an implied decrease in homing abilities displayed by LysM PTP1B mature BMDCs.

LysM PTP1B BMDCs display defects in migration to lymph nodes

Our *ex vivo* and *in vitro* findings which demonstrate that loss of PTP1B in DCs causes defects in DC migration encouraged us to determine a more definitive role for PTP1B in the regulation of DC migration *in vivo*. To this end, we performed an *in vivo* tracking experiment using adoptive transfer (subcutaneous injection) of $CD11b^{+}$ CFSE-labelled BMDCs from control and LysM PTP1B mice into naïve control animals and analysed the BMDC numbers migrating to the draining lymph node by flow cytometry. In agreement with other reports (ref) we found that DCs migrate to the draining lymph node under homeostatic conditions in small numbers as part of the process of maintaining homeostasis and self-tolerance. We present here that in five out of six control mice, small numbers of control BMDCs were detectable in the draining lymph node after inoculation into the skin (Fig. 4). However, a migration defect in LysM PTP1B DCs was observed, since LysM PTP1B DCs were only detected in the draining lymph node in only one out of the six mice (Fig. 4). These

data are in line with our *ex vivo* and *in vitro* results and provide definitive evidence for PTP1B regulatory role in the migration of dendritic cells to regulate the immune function..

PTP1B deficiency in BMDCs decreases podosome number and increases focal contacts.

In migratory DCs, podosome formation is strongly modulated by LPS (West et al., 2004; Svensson et al., 2008b; Yamakita et al., 2011). In the initial hour after LPS exposure, there is a sharp decrease in the number of podosomes, which then recovers and increases over several hours, after which it declines to a level such that, by the time the DC is fully mature, it fails to express podosome structures but instead develops focal contacts (West et al., 2004; Svensson et al., 2008b). We explored the early cell responses of LysM PTP1B BMDCs to LPS, by assessing the number of podosomes in untreated cells and 15 min after LPS pulse. As expected, podosomes appeared as large and defined actin/vinculin rosette-shaped structures in control untreated BMDCs, which disappeared in a high proportion of cells after LPS-treatment (podosome-rich control BMDCs w/o LPS=65%±7 and with LPS=22%±6; $p < 0.005$) (Fig. 5A and B). Interestingly, untreated LysM PTP1B BMDCs constitutively displayed significantly fewer podosomes in comparison to untreated control BMDCs, but exposure to LPS had a lesser effect on decreasing podosome number (Fig. 5A and B). Podosome loss after LPS pulse is accompanied by an increase in the number and extent of vinculin positive focal contacts (West et al., 2004). Untreated LysM PTPIB BMDCs constitutively displayed a 2-fold increase in focal contacts over control BMDCs. In contrast, while LPS treatment induced a 3-fold increase in focal contacts in control DC, LPS treatment of LysM PTP1B BMDCs induced a no significant increase (12.08±2.68 in untreated control vs 33.06± in LPS-treated control BMDCs; *p* value = 0.005; 20.45±4.74 in untreated LysM BMDC vs 29.52±4.27 in LPS-treated LysM-PTP1B BMDCs; *p* value = 0.1) (Fig. 5C). These data indicated that the inverse correlation between podosome number and focal contacts induced in control BMDCs is lost when PTP1B is absent.

One of the hallmarks of activation of DCs with TLR ligands is the rapid recruitment of the actin pool to mediate a transient increase in endocytic activity (West et al., 2004). As expected, FITC-dextran accumulation increased approximately

by 2.5±0.25 fold after treatment of control BMDCs with LPS (Fig. 5D). We found that LysM PTP1B BMDCs displayed a similar two fold increase in endocytosis (x 2.0±0.23; *p*= 0.9 (Fig. 5D).

This indicates that loss of PTP1B does not affect function globally in immature DCs, but selectively modifies the switch from firm adhesion (which takes place during microbial challenge) to the re-appearance of podosomes, allowing the activated DC to leave the peripheral tissues and commence its journey to the SLO.

Podosome defects in LysM PTP1B BMDCs are associated with an increase in phosphorylation of Src at Tyr527 .

ER-bound PTP1B physically interacts with Src kinase (Monteleone et al., 2012) where regulation of its tyrosine kinase activity occurs through control of its phosphorylation status at two major sites: Tyr416/419 in the activation loop, which occurs upon kinase activation; and Tyr527/530 in Src tail, which is responsible for Src auto-inhibition (Bjorge et al., 2000a). PTP1B has been reported to activate Src in several cancer cell lines, HEK 293 cells, L cells and platelets, but not in murine fibroblasts and COS-7 monkey kidney fibroblasts (Arregui et al., 1998; Bjorge et al., 2000b; Cheng et al., 2001; Arias-Salgado et al., 2005; Z. Zhang et al., 2006; Zhu et al., 2007). In BMDCs, loss of PTP1B caused an increase in Tyr527 phosphorylation but had no effect on Tyr418 phosphorylation (Fig. 6A and B). We found that loss of PTP1B is associated with higher levels of Src phospho-Tyr527 under basal conditions and 5 min post-LPS stimulation. However, at later time-points (15 min) the levels of Src pY527 seem to drop, suggesting that PTP1B controls the acute phase of LPS stimulation and that likely other protein tyrosine phosphatases take over at these later stages. Consistent with this view, the phosphate levels of the Src autophosphorylation site, Tyr418, increased by 15 min of LPS stimulation in absence of PTP1B. In addition, LPS-induced phosphorylation of p38 was unaltered between LysM PTP1B and control BMDCs (Fig. 6A). In line with other reports, we also observed incorporation of Src into podosome structures in immature BMDCs, which is disrupted after LPS-treatment (Fig. 6B, see inset).

These findings uncover the mechanism responsible for the podosome defects in LysM PTP1B BMDCs and identify PTP1B as a positive regulator of Src activity in DCs by dephosphorylating the phosphorylation site which maintains Src in an inactive conformation, Tyr527. .

Loss of PTP1B in BMDCs causes defects in DC:T cell contact and T cell priming.

PTP1B levels in T-lymphocytes ($CD4^+$ cells) are unaffected in LysM PTP1B mice (Grant et al., 2014). This allowed us to selectively investigate the role of BMDC PTP1B in the activation of T cells by DCs. Time-lapse imaging of BMDC:T cell co-culture from control mice demonstrated frequent contacts of $CD4^+$ T cells with BMDCs during the first hour (Supplementary video 5) and a markedly reduced number of contacts after 24 h co-culture (Fig. 6A). Strikingly, the average number of T cells establishing contact with a BMDC during the first 1 h of co-culture declined by ~50% when co-culturing BMDCs and T cells from LysM PTP1B mice ($p < 0.002$) (Supplementary video 6 and Fig. 7A). We next measured the duration of the contracts (contact time) in Lys M PTP1B and control co-cultures and found that in co-cultures of control mice over half of the contacts observed took place for more than 30 min, whereas in co-cultures from LysM PTP1B mice most contacts (84%) lasted less than 30 min. (Fig. 7B). Most strikingly, 2/3 of the contacts followed in LysM PTP1B co-cultures occurred for a maximum of 10 min. These findings suggested that LysM PTP1B DCs have a decreased ability to activate T cells. We then investigated whether antigen-specific T cell activation by LysM PTP1B DCs was impaired. For this, we used an OVA-peptide system, which assesses the presentation of the SIINFEKL OVA peptide on MHC class I to the T reporter cell line B3Z (Karttunen and Shastri, 1991). Our data demonstrate that SIINFEKL-pulsed LysM PTP1B BMDCs stimulate T cells less efficiently than similarly-pulsed control BMDCs ($p<0.0001$) (Fig. 8A). In addition, LysM PTP1B mBMDCs had lower expression and surface levels of MHC class I molecules (Fig. 8B). Together, these data support the notion that PTP1B is required for fully functional T cell activation.

Discussion

DCs are the primary sentinels for pathogen recognition and bridge the innate and adaptive immune responses (Steinman, 2007, 2012). In this report we have identified PTP1B as a master regulator of several phases of the immune response elicited by DCs. Specifically, we demonstrate a major role for PTP1B in the conversion of the resting DC from its sessile migratory, immature state to a mature cell, capable of activating naïve T cells in the SLO to become potent effector cells.

The evidence for a central regulatory role of PTP1B in DC physiology is based on the following findings. Here, we show that myeloid-specific deletion of PTP1B leads to: (a) decreased ability of epidermal DCs to migrate from split skin explants *ex vivo* (Fig. 2A); (b) marked decrease in the ability of mature conventional DCs (cDCs) to respond to the lymph node homing chemokine CCL19, while having no effect on the ability of immature cDCs to respond to the pro-inflammatory chemokine CCL3 (Fig. 2B); (c) diminished ability of immature cDCs to dissolve podosomes in response to LPS (Fig. 5A and B), while simultaneously forming increased numbers of focal adhesions (Fig. 5C); (d) decreased DC:T cell contact time related to lowered expression of molecules required for full T cell activation (CD40, CD80 and CD86) as well as MHC Class I and II (Fig. 1D and 7B); and (e) impaired ability of cDCs to present antigen to T cells (Fig. 8A). Thus, PTP1B expression by cDC appears to be necessary for two major components of the immune response: (1) migration of cDCs, and (2) presentation of antigen to the T cell for subsequent activation. LysM PTP1B BMDCs exhibited no reduction in endocytic activity (Fig. 5D) or differences in TLR4 activation and signalling events compared to control BMDCs (Supplementary figures 2 and 3), demonstrating effects primarily on post-processing maturation activities. Importantly, we found that Stat3, a regulator of DC maturation (Nefedova et al., 2004; Nefedova et al., 2005) which is highly phosphorylated in LysM PTP1B macrophages (Grant et al., 2014), appears constitutively and strongly phosphorylated in LysM BMDCs (Fig. 1C).This suggests that PTP1B regulates DC maturation *via* Stat3 dephosphorylation.

The assembly of the podosome structure depends on different sets of intracellular signalling molecules (Svensson et al., 2008a; Kumar et al., 2014). Since podosomes are highly dynamic structures, addition/removal of phosphate groups to/from key components of the podosome machinery may provide the cell with a very efficient mechanism for their rapid turnover as well as for their dynamic attachment / detachment from the substrate. Indeed, many proteins involved in podosome and invadopodia formation are targets of Tyr kinases (Wu et al., 1991; Zhou et al., 2006; Badowski et al., 2008; Macpherson et al., 2012). The action of Tyr kinases is reversed by protein phosphatases; thus it is very likely that Tyr phosphatases play key roles in podosome formation and dynamics. This notion is supported by a growing number of studies showing Tyr phosphatase regulation of podosome and invadopodia formation

(Cortesio et al., 2008; Granot-Attas et al., 2009; Pan et al., 2013; Finkelshtein et al., 2014).

PTP1B is involved in various aspects of T cell function by modulating activity of components of the JAK-STAT pathway (Feldhammer et al., 2013) and is also a physiological regulator of TLR signalling in macrophages (Xu et al., 2008), while preventing myeloid suppressor cell activity in the bone marrow to promote experimental colitis (J. Zhang et al., 2013). Whether these effects of PTP1B are related to the migratory activity of T cells or have a role in invadosome-like protrusion formation is not known. Using the myeloid-cell specific PTP1B ablation model, we provide direct and first evidence for PTP1B regulation of podosome formation and disassembly in immature cDCs. In LysM PTP1B BMDCs, phosphorylation of Src at Tyr527 is constitutively increased and increases further at 5 min but then declines after 15 min of LPS treatment (Fig. 5A). This suggests that Tyr527 phosphorylation of Src is associated with loss of podosomes. Studies with osteoclasts from $Src^{-/-}$ mice demonstrated that podosome number and the podosome-associated actin cloud were decreased in $Src^{-/-}$ osteoclasts while re-expression of Src in $Src^{-/-}$ osteoclasts restored normal podosome organization. In contrast, expression of kinase-dead Src (Src K295M/Y527F) induced the formation of isolated podosomes with little or no actin cloud (Destaing et al., 2008). Here, we demonstrate that LysM PTP1B BMDCs display low podosome numbers, associated with increased phosphorylation levels of Src at Tyr527.. Our data indicate that PTP1B is a modulator of Src kinase activity in BMDCs, by regulating autophosphorylation of Src at Tyr527 thus rendering the enzyme active and promoting optimal podosome turnover.

Furthermore, activation of Src is associated with a shift from stable focal adhesions with actin stress fibers to more dynamic podosome assemblies, regulating cell motility (Sanjay et al., 2001). We demonstrate here that PTP1B LysM BMDCs exhibit an increase in focal adhesions, while having less podosomes, and also display defects in migration towards CCL19. This suggests that when PTP1B is absent in BMDCs, the attachment to the substrate persists and so is stabilised, thus becoming a firm focal adhesion which prevents the podosome from performing its matrix probing / invasive activity. Since it is likely that activation of Src may facilitate the change in cell adhesion complex from focal adhesion to podosome (Sanjay et al., 2001), we envisage a process whereby the adhesive stage of podosome formation occurs during Src-mediated integrin recruitment to the point of cell matrix contact (Gawden-Bone et

al., 2014), thereby forming a transient adhesion which allows the cell sufficient traction for forward propulsion. Subsequently, PTP1B docking with Src in the podosome allows release of the podosome from its attachment and forward migration of the cell, facilitated by the focal release of matrix degrading enzymes. This concept is supported by recent evidence implicating a role for phosphorylation of residues Tyr745 and Tyr752 of β2 integrin in the disassembly of podosomes (Gawden-Bone et al., 2014). Based on our data we envisage the *in vivo* relevance of PTP1B regulation of podosomes in cDCs, whereby PTP1B dephosphorylation of Src facilitates DC release from its relatively firm adhesive attachment to parenchymal cells and tissue components, and further facilitates podosome-mediated probing and invasion of dense tissues such as vascular basement membranes to reach SLO. This notion is supported by studies demonstrating that lymphocytes use podosomes dependent on Src kinase activity to sense the surface of the endothelium and ultimately form transcellular pores (Carman et al., 2007).

We also present data that PTP1B is required for fully competent activation of T cells. LysM PTP1B DCs have decreased numbers and duration of cDC:T cell contacts which are the preamble to T cell activation. Increased duration of cDC:T cell contacts enables the formation of the immunological synapse involving ligand receptor pairings such as CD40:CD40L, ICAM-1:LFA-1 and other accessory molecules surrounding central cores of several MHC Class II peptide:T cell receptor pairings. Recent studies using endothelial cells as antigen presenting cells have shown that the initial T cell contacts or probings of the APC are made by T cell invadosomes as a prelude to the development of the immunological synapse (Sage et al., 2012). Such studies are difficult to directly perform on cDCs due to their highly motile and rugose surface. However, the indirect evidence on DC:T cell contacts in the present study, demonstrates a cDC requirement for PTP1B both for podosome formation as well as sufficient cDC:T cell contacts to mediate antigen specific T cell activation. This suggests that podosome formation is required by both the DC and the T cell to maximise immune synapse formation and T cell activation and that each is regulated separately by its cell-specific PTP1B. This notion is supported by the markedly decreased antigen-specific T cell activation by LysM PTP1B DCs (Fig. 7).

These studies also highlight PTP1B as a potential target for intervention in regulation of the immune response, either when it is out of control (exaggerated) as in a post-infectious state, or is aberrant as in autoimmune disease. Furthermore, immune-

intervention can be rendered cell-specific by targeting either the DC or the T cell and so could be very precisely targeted.

Materials and methods

Animals. All animal procedures were performed under a project license approved by the U.K. Home Office under the Animals (Scientific Procedures) Act 1986 (PPL60/3951). PTP1B $^{fl/fl}$ and LysM PTP1B$^{-/-}$ mice have been described previously (Bence et al., 2006; Grant et al., 2014). DNA extraction and genotyping were performed as described previously (Bence et al., 2006). Age-matched female mice were studied and compared with PTP1B$^{fl/fl}$. Mice were group housed and maintained as in (Grant et al., 2014).

DC culture. DCs were cultured from murine bone marrow as in (Inaba et al., 1992) with modifications. Briefly, bone marrow was flushed from femurs and tibia and cells cultured in 10 cm dishes at 5x10^6/dish in complete RPMI containing 10 ng/ml recombinant GM-CSF (R&D Systems). Medium was replaced on day 2 and replenished with fresh medium on day 4. Cells were generally used on day 6, at which point ~ 70% of cells were CD11c$^+$as assessed by FACS, unless stimulated with 1µg/ml LPS (L4391, Sigma) for 24 h to induce maturation.

Cytokine secretion. To measure cytokine production by immature (no LPS) and mature DCs (LPS 1µg/ml, 24 h, Sigma) cell culture supernatants were collected and analysed for the presence of IL-6, TNF-α, IL-1β, IL-10 and IL-12 using the mouse inflammatory cytometric bead assay kit (BD Biosciences) and FACS Array system (BD Biosciences) according to the manufacturer's instructions.

Immunoblotting. Immunoblots were performed as in (Grant et al., 2014) using antibodies from Cell Signaling (unless stated otherwise), against total and phosphorylated JNK/SAPK T183/Y185 (R&D Systems), total and T202/Y204 phosphorylated ERK1/2 and total and S172 phosphorylated TBK1/NAK, total (against amino-terminus c-Src antigen) and Y418 and Y527 phosphorylated Src, total and phosphorylated T180/Y182 p38, total and phosphorylated Y705 STAT3, SHP2 (Santa Cruz), GAPDH (Abcam) and PTP1B (Millipore).

Microscopy and image analysis. Cells were stained with Rhodamine-phalloidin, PTP1B, total and pY527 Src. Slides were imaged on a Zeiss LSM 700 using a x100 Plan-Apochromat objective (NA 1.46). The primary antibodies are described under Immunoblotting section except the vinculin mouse monocloal antibody which was from Cell Signaling.
Podosomes were stained and quantitated as in (West et al., 2004). Briefly, podosomes were detected by staining fixed cells with Rhodamine-phalloidin (Sigma) and co-stained for vinculin permeabilising the cells with 0.2% TX- 100 before antibody incubation. Images were collected using a Zeiss LSM700 Meta confocal microscope. Podosomes were quantitated on duplicate coverslips by randomly selecting at least 5 fields on each coverslip, each field containing around 10 cells. 5 experiments were performed. The percentage of cells containing clearly visible podosomes was then calculated.

Focal contact/vinculin staining was measured as in West et al 2008 **For quantitation of the number of focal contacts or of the ventral cell surface area, images collected using identical acquisition parameters were analyzed using Volocity software (PerkinElmer). Focal contacts were selected by intensity of green fluorescence (vinculin) and distinguished from podosomes based on size (>10µm^2). The total number of focal contacts for** individual complete cells (defi**ned by regions of interest) in each image was then calculated. For measurement of the ventral surface areas, all o**f the cells in an image were first identified by fl**uorescence in the red channel (**Rhodamine-**phalloidin staining of cortical actin** ALAN IS THIS CORRECT??**). This selected the precise area of the cells. To extract this data** for individual cells in the fi**eld, each complete cell was selected using a simple region of interest. The mean area per cell for each condition was then calculated.**

Flow Cytometry. BMDCs were blocked with CD16/CD32 rat anti mouse Fc Block antibody cocktail (BD Bioscience) and surface stained with antibodies against F4/80 Alexa Fluor 488 (eBioscience), CD11c APC, CD11c Brilliant Violet 450 (eBioscience), CD11b PerCpCy5.5, CD40 FITC, CD86 PE, MHC II I-Ab FITC, MHC I H-2Kb Alexa Flour 647 (Biolegend) and CCR7 PE (Abcam). Antibodies were purchased from BD Biosciences unless otherwise stated. Intracellular staining was performed by fixing cells and permeabilising using BDCellFIX and BD Perm/Wash buffer and stained using F4/80 Alexa Flour 700, CD11c Brilliant Violet 450, MHC I H-2Kb Alexa Flour 647 (all from eBioscience) and CCR7 PE (Abcam). Multi-colour flow cytometry experiments were performed using LSR II analyser (BD Biosciences). Data were analysed with BD FACS Diva and FlowJo software.

Measurement of dextran uptake. FITC-dextran uptake measurement was performed as in (West et al., 2004).

***In vitro* migration assay.** BMDC migration was measured using transwells (8µm pore; Costar). Transwell membranes were coated with collagen overnight and rinsed three times with PBS. 5x10^{5}cells in 400 µl were added in culture medium without chemokines into transwell inserts in 12-well plates. The bottom of the wells contained 1ml of culture medium with 100 ng/mL of either CCL3 or CCL19 (Peprotech), or without chemokines. Cells were incubated for 2.5 h at 37°C. Inserts were removed from the wells and the cells that had migrated to the bottom of the wells were harvested and counted in a Coulter Counter (Beckman Coulter, High Wycombe, UK). Migration was expressed as the ratio between cells migrated in response to chemokines and cells migrated in the absence of chemokine.

***Ex vivo* migration assay.** Epidermal sheet preparations and in situ staining of Langerhans cells (LCs) were done as in (Price et al., 1997). Briefly, ears from culled naive LysM PTP1B and control mice were cut at the base with scissors. The ears were washed twice with PBS and once with 70% ethanol. Ears were spread out on a petri dish, allowed to dry, and then split into dorsal and ventral halves with forceps. The dorsal halves were floated individually on 3 ml of RPMI-FCS containing medium. The explants were incubated at 37°C in a 5% CO2 incubator. After three days explants were removed and epidermal sheets were prepared as described in (Price et al., 1997). Control explants were also established in triplicate. Samples were imaged using a Zeiss 700 confocal microscope with a x40 Plan-Neofluar objective (NA 1.3),

z stacks were collected to cover the whole thickness of the epithelium. The number of LC/microscope field was counted using Volocity software (Perkin-Elmer).

Adhesion assay. Non-treated tissue culture 96 well plates were pre-coated by incubating o/n with collagen (50 µg/ml). Coated wells were rinsed 3x with PBS. $5x10^5$ Immature and mature BMDC were seeded per well of a non-tissue culture treated plate and left to adhere for 30 min and 1h. Non-adherent cells were washed off and adherent cells were stained with crystal violet, washed again 2x in PBS and lysed in 100 µl lysis buffer avoiding formation of air bubbles. OD595 was measured in a plate reader OpsysMR (DYNEX Technologies). Results were normalised by dividing the numbers of adherent cells by the numbers of immature DCs adhering to collagen for 30 min.

Random migration and automated cell tracking. To assess speed of random migration of mature control and LysM PTP1B BMDCs Ibidi µ-slides $VI^{0.4}$ were coated with collagen, washed with medium and seeded with 50µl of BMDCs at a concentration of $2x10^5$ cells/ml. Once the cells had adhered to the slide the slide reservoirs were filled each with 50 µl of medium and sealed with coverslip squares using silicone grease. The slides were then imaged on a Nikon Eclipse Ti microscope using objective ×20 phase with images taken every 2 min by a Photometrics cascade II CCD (charge-coupled device) camera using Nikon NIS Elements software. Cells were maintained at 37^oC and 5% CO_2 in air in a cage incubator (Okolab). Time-lapse images from all slide channels were collected under the same conditions using the multi-point visiting stage facility. Information about cell displacement was extracted from time-lapse images using an automated procedure written in Matlab. The pre-processing was done using the open source software ImageJ. For the segmentation, the “Gaussian Blur” filter is applied with a radius of 2 pixels followed by “Unsharp Mask” filter, radius 10 and weight 0.7 to 0.9. The pre-processing is continued by adjusting manually the brightness and contrast, binarization and filling the holes. A segmentation algorithm used by Blair and Dufresne is included in the automatic procedure (http://physics.georgetown.edu/matlab/tutorial.html). For the actual tracking software the scheme proposed by Nicholas Darnton (http://www.rowland.harvard.edu/labs/bacteria/index.php) was used but the procedure was changed in order to use the area of intersection as the main criterion for cell identification rather than the distance only as in the initial software. Once the tracks are extracted, for each step the total displacement of each cell is calculated as well as the displacements on the x and y axes. On each frame at least 15 cells were tracked simultaneously. The data were divided into bins, corresponding to one-hour intervals and averages and standard errors of the mean were computed for each interval after discarding all null values. Speeds of random migration are expressed as µm/sec.

In vivo tracking experiment

Naïve female C57BL/6 mice aged 11-12 weeks were used for adoptive transfer. Mice were provided and housed under pathogen-free conditions at the Medical Research Facility of University of Aberdeen.BMDCs from control and LysM PTP1B mice were prepared as explained above. BMDCs were harvested on day 6 of culture. Cells were stained with CFSE (Life technologies) at a working concentration of 5 µM for 15 min at 37˚C before centrifuged and resuspended in pre-warmed PBS for further 30 min

incubation. Cells were then washed and counted. Each mouse received 2 x 10^6 of the above CFSE labelled wt or PTP1B LysM BMDCs subcutaneously in a volume of 100 µl. Draining lymph nodes (DLNs) were harvested 24 hour post injection and processed to single cells suspension. Migrated CFSE labelled BMDCs with the DLNs were analysed by flow cytometry. Absolute number of migrated CFSE labelled BMDCs were calculated as follows: (CFSE positive cells acquired/total leukocyte population acquired) x LN cell count.

Isolation of T-cells. Isolation of $CD4^+$ T cells was carried out as in (Grant et al., 2014).

DC:T cell contact assay. BMDCs from control and LysM PTP1B mice were generated as explained above. Coverslips coated with $3x10^5$ DCs were placed into a chamber on the microscope at 37°C in a 5% CO2 atmosphere. DIC images were acquired using a Zeiss Axiovert 100 (Jena, Germany).Images were collected every 30 sec for 1 h starting after the addition of the same number of T cells ($3x10^5$) (t=0). The duration of the contacts established by single T cells was assessed as in (Benvenuti et al., 2004). For this equal numbers of contacts were followed for 1 h in control and LysM PTP1B co-cultures.

Antigen presentation assay. Assays were performed as in (West et al., 2004). BMDC were seeded in triplicate in 96 well plates at a density of $5x10^4$ cells per well in cRPMI. Cells were left to adhere and then incubated with serial dilutions of SIINFEKL peptide for 6 hours. Cells were then washed twice with PBS and fixed with 0.05% gluteraldehyde diluted in PBS, and then quenched by adding 0.2M glycine diluted in PBS. Cells were then washed and incubated with $5x10^4$ B3Z cells and incubated for 16 hours. Stimulation of the B3Z hybridoma (ref) was measured by luminescent ß-galactosidase assay following manufacturer's instructions (Clontech).

Statistical analysis. Data are expressed as mean of all experiments $\pm$SEM. N is indicated in the figure legends. Statistical analyses were performed from all available data using two-tailed Student's t tests. *P* values are shown in figures when data are considered significant ($p < 0.05$).

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Figure Legends

Figure 1 PTP1B loss in BMDCs causes increased phosphorylation of Stat3 and low expression of DC maturation markers. **A**: PTP1B levels are shown in BMDCs from LysMPTP1B and control littermates. SHP2 and GAPDH loading controls are shown. **B** Confirmed PTP1B loss by immunocytochemistry. PTP1B levels (green) in control immature BMDCs (iBMDC) and mature BMDC (mBMDCs). All images were taken at the same microscope settings. Representative images are shown. Scale bar: 20 µm. **C Left:** LysM PTP1B BMDCs display increased basal and LPS-induced phosphorylation of STAT3 (Y705). Representative Western blots shown. Right: Quantification of band intensities expressed as Phospho/Total Stat3 ratio (N=4). **D** Loss of BMDC PTP1B causes defects in expression of maturation markers and co-stimulatory molecules. DCs were analysed after gating on double positive $CD11c^+$ and $CD11b^+$ population. Histograms were obtained by FACS. $CD11c^+CD11b^+$ double positive population has been gated based on the unstained sample control. Bar charts summarise the data displayed on the histograms (N=3 in triplicates±SEM).

Figure 2: Loss of PTP1B in BMDCs causes defects in *ex vivo* migration and chemotaxis towards CCL19. **A** LysM PTP1B LC migrate less into the medium. Left: LC from control ear split explants (w/o migration induction) and LC from ear split explants incubated in medium for three days to induce migration (after migration) are shown by staining cells with a MHC class II antibody. Representative images shown. Scale bar: 50 µm. Right: Diagram shows LC/field comparing control and LysMPTP1B ear split explants. Data from dorsal and ventral ear splits were pooled and expressed as mean. N=16 (control) and N=12 (LysM PTP1B). **B** Transwell assay of iBMDC and mBMDC from control and LysM PTP1B BMDCs towards CCL3 (left panel) and CCL19 (right panel) (N=4 in triplicates±SEM). **C** Representative histograms (N=3) showing low CCR7 expression in LysM PTP1B BMDCs were obtained by FACS.

Figure 3: Loss of PTP1B increases BMDC adhesion to collagen. **A** iBMDC and mBMDC from LysM PTP1B and control mice were left to adhere for 30 min and 1 h

in 96 well collagen-coated plates and photographed after staining with crystal violet. Representative photo of stained adherent cells is shown. **B** Fold adherence is measured as indicated in materials and methods by measuring OD595 (N=3 in triplicates±SEM).

Figure 4:

Figure 5: LysM PTP1B BMDCs display less podosomes, more focal contacts and unaltered antigen uptake. **A** Podosomes are revealed by rhodamine phalloidin and vinculin staining. Cells with podosomes are marked with arrows. 15 min LPS treatment induces podosome loss. All images were taken at the same microscope settings. Scale bar: 20 µm. **B** Quantification of cells with podosomes in LysM PTP1B and control BMDCs. Plus and minus signs denote LPS-treatment or lack thereof (respectively). 50 cells were examined per experiment (N=5±SEM). **C** Analysis of the area of vinculin in LPS-treated and non-ttreated BMDCs. At least 45 cells were scored (N=3±SEM). **D** Antigen uptake competence by measuring FITC-conjugated dextran uptake. Bar diagram shows the fold change in MFI; N=3±SEM.

Figure 6: LysM PTP1B BMDCs have increased levels pY527 Src. **A** Representative Western blots of Src and p38 phosphorylation in iBMDC after 5 and 15 min LPS. PTP1B loss,SHP2 and GAPDH levels are shown as controls. N=3. **B** Src localises to podosome-like puncta (see red staining for Src in inset) in untreated control iBMDC. LysM PTP1B BMDCs display increased pY527 Src (in green) levels. Nuclei are stained with DAPI. LPS treatment was 5 min. Scale bar: 20 µm.

Figure 7: Loss of PTP1B in BMDC causes defects in DC:T cell contact (supplementary videos 5 and 6). DC:T cell co-cultures from LysM PTP1B mice display fewer contacts (**A**) and for a shorter time (**B**) than co-cultures from control mice (N=3).

Figure 8: A Beta-galactosidase assay with B3Z cells shows that LysM PTP1B BMDCs are not able to activate a T reporter cell line to the same extent as control BMDCs (N=3 in triplicates). The OVA SIINFEKL peptide was added at different concentrations. **B** Loss of BMDC PTP1B causes defects in intracellular and surface

expression of MHC class I. Representative histograms obtained by FACS shown. Bar charts summarise the data displayed on the histograms (N=3 in triplicates).