Biochemical and Biophysical Research Communications **286,** 184–188 (2001)
doi:10.1006/bbrc.2001.5360, available online at http://www.idealibrary.com on IDEAL®

Functional Abnormalities in Protein Tyrosine Phosphatase ε-Deficient Macrophages

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Received June 28, 2001

Protein tyrosine phosphatase ε (PTPε)-deficient mice were generated by targeted deletion of exons 3, 4, and 5 of the *Ptpre* gene. Mice homozygous for this deletion (*Ptpre*$^{\Delta 3\text{-}5}$) were fertile, bred and developed normally and exhibited no overt phenotype. However, closer examination of the function of macrophages from these mice revealed a defect in the regulation of the respiratory burst. While bacterial lipopolysaccharide (LPS) or tumour necrosis factor α (TNFα) were able to prime bone marrow-derived macrophages (BMM) from wild type (*Ptpre*$^{+}$) macrophages for an enhanced respiratory burst, they were unable to do so in macrophages from PTPε-deficient mice. PTPε-deficient BMM also had abnormalities in cytokine production with a reduced ability to produce TNFα and enhanced IL-10 production in response to challenge with LPS. These findings suggest an important role for PTPε in the control of macrophage function.

Key Words: **protein tyrosine phosphatase; PTPε; knockout; macrophage; respiratory burst; priming; LPS; TNFα; IL-10.**

Tyrosine phosphorylation is a reversible process with the net level of tyrosine phosphorylation being the result of a dynamic balance between the activities of the tyrosine kinases, which phosphorylate proteins on tyrosine residues and the protein tyrosine phosphatases (PTPs) which dephosphorylate phosphotyrosine residues. However, PTPs do not necessarily simply oppose the action of the tyrosine kinases. As kinases can themselves be regulated by tyrosine phosphorylation, PTPs can potentially act as both positive and negative regulators of tyrosine phosphorylation (1, 2).

PTPs comprise a large family of related molecules that can be broadly classified into two main groups; the transmembrane and cytoplasmic (3, 4). The sometimes large, receptor-like, extracellular portions of the transmembrane PTPs suggest they may be regulated by extracellular ligands (5). The mechanism(s) of activation of the cytoplasmic PTPs is not clear.

PTPε was originally identified as a receptor-like protein tyrosine phosphatase most closely related to PTPα (6). A cytoplasmic isoform of PTPε, that is identical to the cytoplasmic portion of the transmembranal isoform yet having a distinct N-terminus, has also been identified (7, 8). Recently, two further cytoplasmic forms of PTPε were identified. One arising from an alternate translation initiation site within PTPε mRNA transcripts and the other arising due to proteolytic processing of the larger molecules (9).

Both the transmembranal and cytoplasmic isoforms of PTPε (PTPε-M and PTPε-C, respectively) are transcribed from a single gene (*Ptpre*) located on mouse chromosome 7 and in a syntenic region on human chromosome 10q26 (10, 11). However, by using different promoters, the two isoforms have differing expression patterns with PTPε-M found predominantly in brain, testes and lung while PTPε-C is expressed primarily in hematopoietic tissues (7, 12). Furthermore, the levels of PTPε-C, but not PTPε-M, increase during differentiation and/or activation of murine macrophages and human HL-60 cells (7, 12).

We have used the Cre/Lox system for the targeted disruption of the *Ptpre* gene in mice and show that PTPε-deficient macrophages have abnormalities in the

Abbreviations used: BMM, bone marrow-derived macrophages; IL-10, interleukin-10; LPS, lipopolysaccharide; PMA, phorbol myristate acetate; PTP, protein tyrosine phosphatase, TNFα, tumour necrosis factor α.

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0006-291X/01 $35.00

regulation of the respiratory burst and the production of cytokines in response to bacterial lipopolysaccharide (LPS).

MATERIALS AND METHODS

Generation of the PTPε-deficient mice. A *Ptpre* genomic fragment was isolated from a 129SvJ genomic liver DNA library (Stratagene, La Jolla, CA). This clone was shown to contain exons 3–6 of mouse *Ptpre* as well as the promoter for the cytoplasmic isoform (12). Exon 3 corresponds to the transmembrane domain of the receptor-like PTPε, exon 4 is the first exon of the cytoplasmic isoform and contains the initiating methionine for this form. Exon 5 is the first common exon used by both the cytoplasmic and the receptor-like PTPε. Three fragments derived from the *Ptpre* genomic DNA were sequentially cloned into the pFlox vector, kindly provided by J Marth (13), to create the final targeting vector, pFXe2. First, the ~4.5-kb *Hin*dIII fragment containing exon 6 was cloned into the *Hin*dIII site of the pFlox vector to provide the long arm for homologous recombination. The *Hin*dIII/*Eco*RI fragment containing exons 3–5 was cloned into the unique *Bam*HI site of pFX4.5. The ~1.2-kb *Stu*I/*Eco*RI fragment was cloned in the correct orientation into the unique *Sal*I site of pFXB to create the completed targeting vector.

The *Not*I-linearized targeting vector was electroporated into R1 embryonic stem cells (14). Six independent G418-positive $Ptpre^{flox}$ embryonic stem cell clones identified by PCR were confirmed to be homologous recombinants by Southern blotting. The Cre recombinase expression plasmid, pIC-Cre (kindly provided by Dr. K. Rajewsky (15)) was transiently transfected into the positive $Ptpre^{flox}$ embryonic stem cell clones and the $Ptpre^{\Delta 3\text{-}5}$-mutant embryonic stem cell clones were confirmed by Southern blot analysis. *Ptpre* targeted embryonic stem cells were microinjectioned into 3.5-day-old blastocysts harvested from C57Bl/6 mice. Chimeras generated from this procedure were bred for germline transmission.

RNA extraction and PCR analysis. Brain and spleen were dissected from wild type ($Ptpre^{+}$) and PTPε-deficient ($Ptpre^{\Delta 3\text{-}5}$) mice and total RNA extracted by a single-step acid guanidium thiocyanate-phenol–chloroform method (16). RNA samples were treated with RQ1 RNase-free DNase (5 U, Promega Corporation, Madison, WI) to remove any DNA. cDNA was synthesized from 2 μg of each total RNA sample using MMLV reverse transcriptase enzyme (200 U, Promega) and an oligo $(dT)_{15}$ primer (0.05 μg/μl, Promega). Negative controls were included in which reverse transcriptase enzyme was replaced with water.

PCR amplification of cDNA was carried out in a 25 μl reaction containing 2 μl of cDNA, 4 ng/μl each of forward and reverse primers, 1.25 U *Taq* DNA polymerase (Promega), 400 μM of each dNTPs and 2 mM $MgCl_2$ for 35 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 1 min. PCR products separated by electrophoresis and transferred to a nitrocellulose membrane (Hybond N^{+}, Amersham Pharmacia Biotech, Sydney, Australia) overnight by capillary action in 0.4 M NaOH. The membranes were probed with an internal oligonucleotide labeled with [γ-^{32}P]ATP (2 mCi/ml, NEN Research Products, Boston, MA) and bands visualized by exposure to X-ray film.

Bone marrow-derived macrophages (BMM). Murine BMM were prepared by the culture of precursor cells, isolated from the bone marrow of mice and cultured in RPMI 1640, 15% (v/v) fetal calf serum (CSL Limited, Parkville, Victoria, Australia) and 30% (v/v) L-cell-conditioned medium (a crude source of the macrophage-specific growth factor CSF-1), as previously described (17).

MTT assay. Cell growth was assessed using the MTT dye assay described by Hansen *et al.* (18).

Respiratory burst. The respiratory burst of adherent macrophages was assessed by the phorbol myristate acetate (PMA)-induced reduction of cytochrome *c* (17). The cells, grown in 24-well tissue culture plates, were incubated in 1 ml of phosphate buffer (121 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 3.1 mM NaH_2PO_4, 12.5 mM Na_2HPO_4 and 11 mM glucose, pH 7.3), containing 80 μM cytochrome c with and without 1 μM PMA for 90 min at 37°C. The supernates were then collected, and the absorbance was read at 550 nM. After removal of the cytochrome *c* reaction medium, adherent cells were washed with phosphate-buffered saline and the total protein in each culture well was determined by the Lowry method using bovine serum albumin as a standard. The results were expressed as nmol of cytochrome *c* reduced per milligram of cell protein. All experiments consisted of triplicate determinations.

Tumour necrosis factor α (TNFα) and interleukin-10 (IL-10) assays. BMM were incubated with (or without) 10 μg/ml LPS for 24 h. The culture supernates were then collected, clarified by centrifugation (5 min, 15,000 rpm, 4°C) and stored at −20°C until assayed. TNFα and IL-10 levels were measured by ELISA using monoclonal antibodies to human TNFα and to human IL-10 (PharMingen, San Diego, CA) (19). The assays were sensitive to >40 pg/ml for both TNFα and IL-10.

RESULTS AND DISCUSSION

PTPε-deficient mice were created by using the Cre/Lox system to delete exons 3, 4, and 5 of the PTPε gene. The absence of PTPε gene expression in mice homozygous for this deletion ($Ptpre^{\Delta 3\text{-}5}$) was confirmed by RT-PCR (Fig. 1). Some truncated transcripts of PTPε-M, consistent with the loss of exons 3 and 5, were detected in brain tissue from $Ptpre^{\Delta 3\text{-}5}$ mice (Fig. 1B). Although the loss of exons 3 and 5 results in an in-frame deletion thus producing transcripts of PTPε-M which, if translated, could possibly produce an active protein, it is unlikely that any such protein would be retained in the cell. Receptor type PTPs contain a signal sequence that directs the protein to the extracellular matrix. The secretion of the protein is prevented by the presence of a transmembrane domain (4) resulting in the expression of the protein on the cell surface. Since the transmembrane domain of PTPε-M is located in the deleted exon 3, any protein produced from the truncated transcripts of $Ptpre^{\Delta 3\text{-}5}$ mice would be expected to be secreted from the cell due to the presence of the signal sequence coded for by exon 2. Recently, Gil-Henn *et al.* have reported novel cytoplasmic forms of PTPε that can be produced from PTPε-M or PTPε-C mRNA transcripts by using an alternate internal translation initiation site (9). However, this alternate initiation site is located within the deleted exon 5 and thus these novel products would not be expected in the $Ptpre^{\Delta 3\text{-}5}$ homozygous mice. Similarly, PTPε-C would also not be expected to be expressed in these mice as the deletion of exon 4 removes both the promoter region and the initiating methionine of PTPε-C. Importantly for this study, PTPε-M is not normally expressed in macrophages (12) and the use of PCR-primers common to both PTPε-M and PTPε-C downstream of the deletion (primers D-E in Fig. 1A) failed to amplify any product from the macrophage-rich spleen tissue (Fig. 1B), confirming that PTPε is not expressed in macrophages from $Ptpre^{\Delta 3\text{-}5}$ mice.

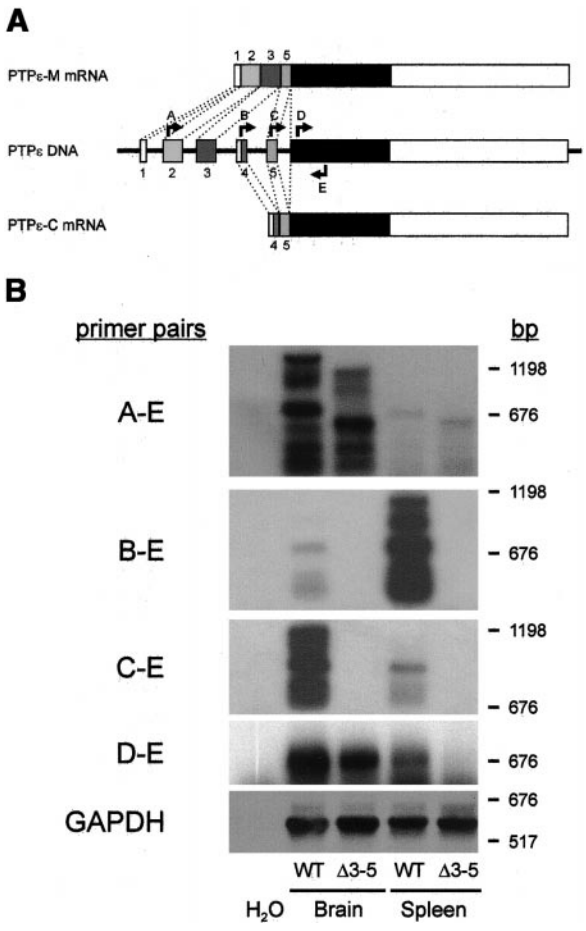

FIG. 1. Expression of PTPε mRNA transcripts in brain and spleen of *Ptpre*$^{+}$ and *Ptpre*$^{\Delta 3\text{-}5}$ mice. (A) Diagrammatic representation of the structure of the PTPε gene and the PTPε-transmembranal (PTPε-M) and cytoplasmic (PTPε-C) mRNA splice variants. Exons 1–5 are indicated (numbers) with the open (unshaded) regions representing untranslated sequence. Intron/exon boundaries beyond exon 5 are not shown. The position of forward primers A (5′-ATG GAG CCC TTG TGT CCA CTC CT-3′), B (5′-AGC TCA GCC ATG AGC AGC AG-3′), C (5′-GGA AGG CCG TGG TCA GCA GC-3′), and D (5′-AGC AAA GGT CAG CCA CCA TCG-3′) and the reverse primer, E (5′-CAC TAG CTA CGG TAC TAC CTG-3′) used in B are also shown (arrows). Diagram is not to scale. (B) RNA isolated from brain and spleen of *Ptpre*$^{+}$ and *Ptpre*$^{\Delta 3\text{-}5}$ mice was reverse transcribed into cDNA and amplified by PCR using the combinations of primers indicated. GAPDH transcripts were amplified using specific primers (forward 5′-ACC ACC ATG GAG AAG GC-3′; reverse 5′-CTC AGT GTA GCC CAG GAT GC-3′) as a control for RNA integrity. Transcripts were separated on a 1.5% TAE agarose gel, transferred to a nitrocellulose membrane, and then probed with a ^{32}P-end-labeled internal primer (primer D). GAPDH transcripts were probed using an [α-^{32}P]ATP random primed 1.1-kb *Pst*I fragment of the rat *GAPDH* gene. Results shown are representative of two or more independent experiments.

Ptpre$^{\Delta 3\text{-}5}$ mice were fertile, bred and developed normally and exhibited no gross phenotypic abnormalities. All organs were present and appeared normal at the macroscopic and light microscopic levels. X-ray analysis failed to reveal any abnormalities in the skeletal system and there were no major differences between wild type (*Ptpre*$^{+}$) and *Ptpre*$^{\Delta 3\text{-}5}$ mice detected in blood cell counts and differentials or in blood chemistry (data not shown).

PTPε mRNA is known to be expressed in murine macrophages and expression is enhanced during activation with LPS (12). To investigate more closely the function of macrophages deficient in PTPε, we prepared BMM from *Ptpre*$^{+}$ and *Ptpre*$^{\Delta 3\text{-}5}$ mice. Normal numbers of BMM progenitors were harvested from the bone marrow of *Ptpre*$^{\Delta 3\text{-}5}$ mice and the cells, when grown in CSF-1-rich L

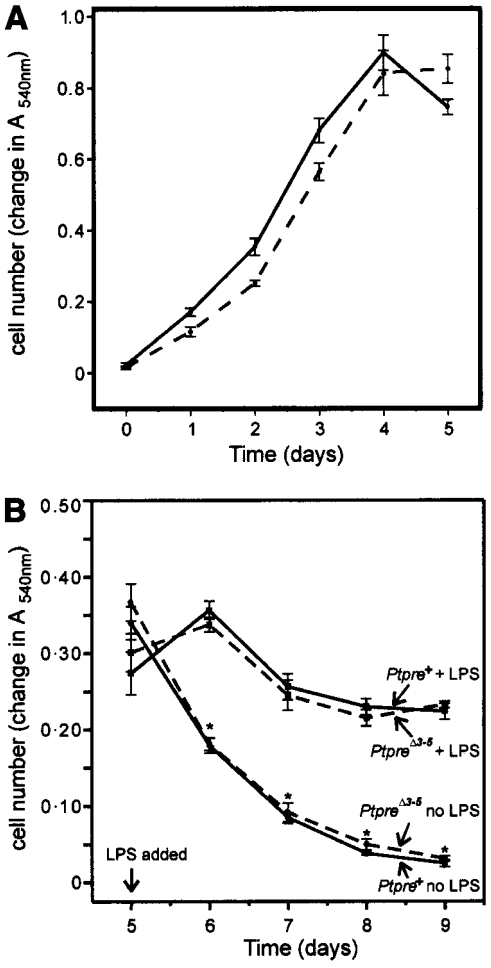

FIG. 2. Proliferation and LPS-induced cell survival in *Ptpre*$^{+}$ and *Ptpre*$^{\Delta 3\text{-}5}$ BMMs. (A) BMM precursor cells from the femurs of *Ptpre*$^{+}$ (solid lines) and *Ptpre*$^{\Delta 3\text{-}5}$ (broken lines) mice were initially seeded into wells at 2×10^{4} cells/well and cultured in the presence of L-cell-conditioned medium (a source of CSF-1) for 5 days. MTT assays were carried out on a daily basis to monitor cell population growth. Means ± SEM of triplicates from a single experiment representative of 3 independent experiments conducted are shown. (B) BMM were cultured in the presence of L-cell-conditioned medium for 5 days. The L-cell-conditioned medium was then withdrawn and cells cultured in the absence or presence of 100 ng/ml LPS. Cell survival was monitored daily using MTT assay to assess cell number. Means ± SEM of triplicates from a single experiment representative of three independent experiments conducted are shown. Asterisks indicate significant decreases ($P < 0.05$, t test) in cell number compared to the number present on day 5.

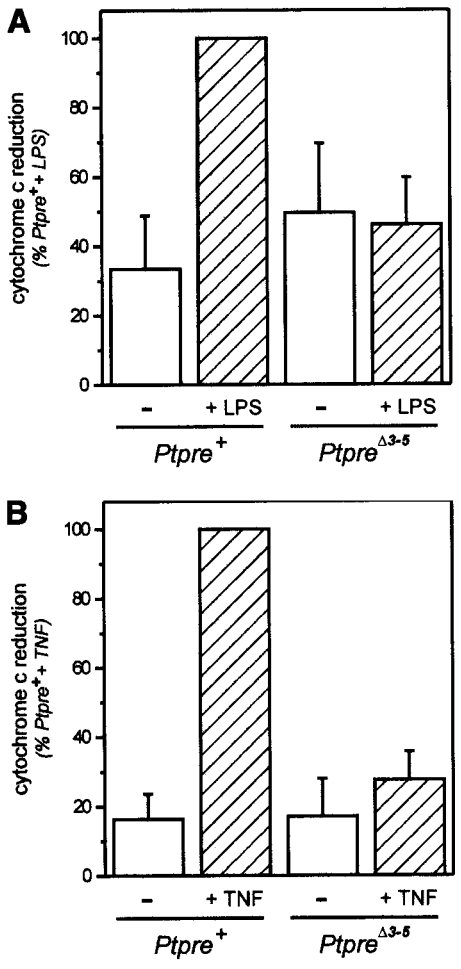

FIG. 3. PMA-induced respiratory burst activity in *Ptpre*$^{+}$ and *Ptpre*$^{\Delta 3\text{-}5}$ BMM. *Ptpre*$^{+}$ and *Ptpre*$^{\Delta 3\text{-}5}$ BMM were treated with tissue culture medium alone or tissue culture medium containing (A) 100 ng/ml LPS or (B) 2 nM TNFα for 48 h. BMMs were then stimulated with 10^{-6} M PMA in the presence of 1 mg/ml cytochrome *c* and the absorbance at 550 nm measured after 90 min. Results are expressed as percent activity of LPS or TNF pretreated *Ptpre*$^{+}$ BMM and shown as the mean ± SEM of four independent experiments. 100% represents 43.7 ± 10.9 and 267.8 ± 52.5 nmol cytochrome *c* reduced/mg protein/90 min (mean ± SEM, $n = 4$), in A and B, respectively.

cell-conditioned medium, proliferated at a similar rate to the *Ptpre*$^{+}$ controls (Fig. 2A). Consistent with our previous reports (17, 20), pretreatment of *Ptpre*$^{+}$ BMM for 48 h with 100 ng/ml LPS or 2 nM TNFα resulted in an increased respiratory burst when subsequently stimulated with PMA. However, both LPS and TNFα failed to enhance the respiratory burst response of *Ptpre*$^{\Delta 3\text{-}5}$ BMM (Fig. 3). We also observed that the LPS-induced production of TNFα was significantly reduced in *Ptpre*$^{\Delta 3\text{-}5}$ BMM (Fig. 4A). However, not all LPS-induced responses are similarly affected. The ability of LPS to maintain cell survival in the absence of CSF-1 was not affected in the *Ptpre*$^{\Delta 3\text{-}5}$ BMM (Fig. 2B) while LPS-stimulated IL-10 production was significantly enhanced in the *Ptpre*$^{\Delta 3\text{-}5}$ BMM (Fig. 4B).

The specific mechanism by which loss of PTPε leads to these abnormalities in macrophage function is not clear. A generalized disruption to the signaling pathways activated by LPS and TNFα would seem unlikely as multiple functions are differentially affected. Our finding that LPS and TNFα are unable to prime the respiratory burst in macrophages from *Ptpre*$^{\Delta 3\text{-}5}$ mice may indicate a more specific role of PTPε in the regulatory mechanisms controlling the respiratory burst. This is consistent with previous work from our laboratory demonstrating a close correlation between the induction of increased PTP activity and the priming of the macrophage respiratory burst (20). However, although LPS and TNFα failed to prime the respiratory burst of *Ptpre*$^{\Delta 3\text{-}5}$ macrophages, under the same conditions, both agents did induce an increase in total PTP activity in *Ptpre*$^{\Delta 3\text{-}5}$ BMM (data not shown) similar to

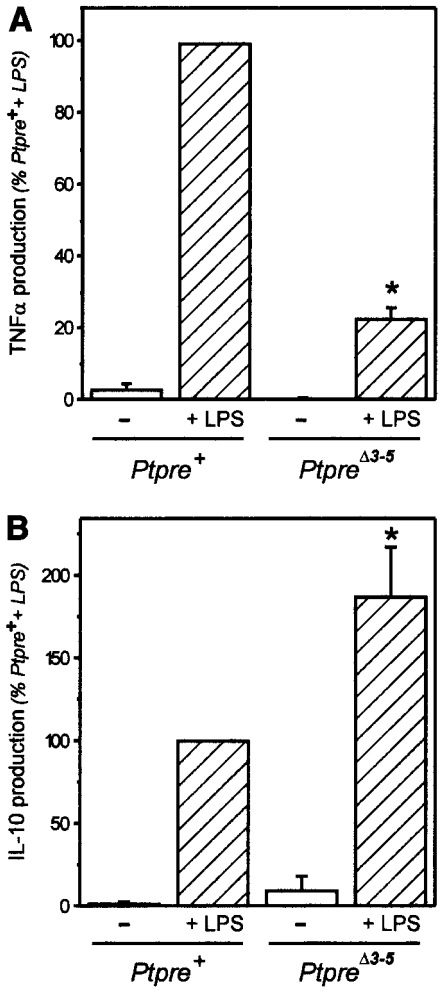

FIG. 4. TNFα and IL-10 production in *Ptpre*$^{+}$ and *Ptpre*$^{\Delta 3\text{-}5}$ BMM. *Ptpre*$^{+}$ and *Ptpre*$^{\Delta 3\text{-}5}$ BMMs were treated without and with LPS (10 μg/ml, 24 h) (hatched) and the supernates assayed for (A) TNFα and (B) IL-10 activity using *in vitro* ELISAs. Results shown are expressed as % LPS-induced activity in *Ptpre*$^{+}$ BMM and the data presented as mean ± SEM ($n = 3$). Asterisks indicate significantly different to *Ptpre*$^{+}$ ($P < 0.05$, 2-way ANOVA).

that reported by Bassal *et al.* (20). This would suggest that PTPε is not responsible for the LPS/TNFα-induced PTP activity and leaves open the question as to the identity of the PTP induced by these agents.

It is possible that the inability to prime the respiratory burst in PTPε-deficient BMM could be an indirect effect related to disruption of cytokine production. IL-10 is known to suppress both the respiratory burst (21) and TNFα production (22) in macrophages and thus it is possible that increased levels of IL-10 produced by *Ptpre*$^{\Delta 3\text{-}5}$ BMM could be feeding-back and inhibiting respiratory burst activity and TNFα production.

Although we can demonstrate significant abnormalities in macrophage function in *Ptpre*$^{\Delta 3\text{-}5}$ mice, these animals do not have an obvious phenotype. This result is consistent with that of Peretz *et al.* who recently reported only early post-natal hypomyelination of sciatic nerve axons in mice lacking PTPε (23). However, as both the respiratory burst and the cytokines TNFα and IL-10 are known to play important roles in inflammation and host defense, it is possible that a more overt phenotype may emerge in mice undergoing an active infection or inflammatory challenge.

ACKNOWLEDGMENTS

This work was supported, in part, by grants from the National Health and Medical Research Council (NHMRC) of Australia (W.A.P.) and the Arthritis Foundation of Australia (W.A.P. and E.V.). S.P. was generously supported through a doctoral studentship from the Medical Research Council of Canada (now the Canadian Institutes of Health Research). The authors thank Jamey Marth for providing the targeting vector backbone pFlox and Klaus Rajewski for providing pIC-Cre recombinase expression plasmid. The authors also give a special thanks to Frank Jirik for his generosity in providing the resources and support that led to the production of the *Ptpre*$^{\Delta 3\text{-}5}$ mice.

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