Two related protein tyrosine phosphatases, TC-PTP and PTP-1B, in the regulation of macrophage development and activation

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A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Your file Votre référence
ISBN: 978-0-494-25171-3
Our file Notre référence
ISBN: 978-0-494-25171-3

1 ABSTRACT

T Cell Protein Tyrosine Phosphatase (TC-PTP) and Protein Tyrosine Phosphatase (PTP)-1B are two closely related enzymes involved in the regulation of cytokine signalling. While tcptp-/- mice display a wide range of hematopoietic defects and die within three to five weeks after birth, ptp1b-/- mice are healthy and show no apparent immune phenotype; nevertheless, in vitro results suggest that both enzymes negatively regulate interferon signalling. The goal of my thesis was to determine their role in macrophages whose activation and maturation is largely dependent on interferon-gamma.

In the first instance, we detected a progressive mononuclear cellular infiltrate in several organs of the TC-PTP deficient mice, which, together with active cytokine production, was likely to be the cause of tissue damage and ultimately their death. Moreover, the tcptp-/- mice showed increased sensitivity to exogenous lipopolysaccharide in vivo and developed symptoms of septic shock. tcptp-/- spleen-derived macrophages were also highly sensitive to lipopolysaccharide. These findings indicated that TC-PTP was necessary for the regulation of inflammatory disease.

In the second part, although ptp1b-/- bone marrow presented no abnormalities at the myeloid progenitor level, it contained more colony stimulating factor (CSF)-1 -responsive cells and the CSF-1 receptor was hyperphosphorylated in ptp1b-/- bone marrow-derived macrophages. ptp1b-/- splenic macrophages also displayed upregulated activation markers as well as increased sensitivity to LPS. Collectively, these results indicated that PTP-1B regulated myeloid differentiation and macrophage activation in vivo.

Lastly, we wished to delete one or both copies of PTP-1B in tcptp-/- and tcptp+/- mice by interbreeding to study the redundancy of the two enzymes. Our results indicated that the double mutant was lethal at a relatively early stage of embryonic development. Mice heterozygous for TC-PTP on the ptp1b-/- background developed symptoms similar to the tcptp-/- mice, and their macrophages were highly sensitive to interferon-gamma as shown by increased Stat1 phosphorylation, indicating a nonredundant role for PTP-1B and TC-PTP in the regulation of interferon signalling.

2 RÉSUMÉ

T Cell Protein Tyrosine Phosphatase (TC-PTP) et Protein Tyrosine Phosphatase (PTP)-1B sont deux enzymes hautement similaires au niveau structural, et impliquées dans la régulation des signaux de cytokines. Les souris *tcptp*$^{-/-}$ meurent tôt après la naissance et présentent plusieurs défauts de développement du système immunitaire. Par contre, les souris *ptp1b*$^{-/-}$ sont viables et ne présentent pas de phénotype immunologique apparent. Malgré cela, les résultats *in vitro* suggèrent que les deux enzymes puissent réguler les voies de signalisation d'interférons. Le but de mes travaux était d'évaluer leur rôle dans les macrophages, dont l'activation et la maturation sont dépendantes de l'interféron gamma.

En premier lieu, nous avons détecté une infiltration progressive des cellules mononucléaires dans plusieurs organes, y compris le cœur. En plus de la production active des médiateurs inflammatoires, ces infiltrations cellulaires étaient probablement responsables des dommages tissulaires et éventuellement de la mort des souris *tcptp*$^{-/-}$. D'ailleurs, les souris étaient plus susceptibles à l'endotoxine et développaient des symptômes de choc septique. Les macrophages dérivés de la rate *tcptp*$^{-/-}$ présentaient également une susceptibilité accrue à l'endotoxine. Ces résultats indiquent que TC-PTP est nécessaire pour le contrôle de la réaction inflammatoire.

Dans la deuxième partie, malgré le fait que la moelle osseuse *ptp1b*$^{-/-}$ contenait un niveau normal de précurseurs myéloïdes, il y avait plus de cellules réceptives à colony stimulating factor (CSF)-1. Le récepteur CSF-1 était également hyperphosphorylé dans les macrophages dérivés de la moelle *ptp1b*$^{-/-}$. Les macrophages de la rate *ptp1b*$^{-/-}$ paraissaient activés d'après l'exposition des marqueurs de surface et par une hypersensibilité à l'endotoxine. Ensemble, ces données démontrent que PTP-1B est un régulateur de la différenciation myéloïde et de l'activation des macrophages.

En dernier lieu, nous voulions étudier les conséquences de l'ablation de l'une ou des deux copies du gène PTP-1B dans les souris *tcptp*$^{-/-}$ et *tcptp*$^{+/-}$. Le but de cette expérience était de déterminer si les enzymes possédaient une fonction similaire. Nos résultats démontrent qu'au moins une copie de PTP-1B ou TC-PTP était nécessaire au développement embryonnaire. Les souris *tcptp*$^{+/-}$*ptp1b*$^{-/-}$ développaient des symptômes similaires aux souris *tcptp*$^{-/-}$, et leurs macrophages étaient extrêmement susceptibles à

l'interféron gamma, susceptibilité indiquée par une augmentation de la phosphorylation de Stat1. Cela démontre que PTP-1B et TC-PTP possèdent un rôle non similaire dans la régulation des voies de signalisation d'interférons.

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AML	Acute myeloblastic leukemia
APC	Allophycocyanin
APC	Antigen presenting cell
BCR	B cell receptor
BMI	Body mass index
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CIS	Cytokine-induced SH2 protein
CLL	Chronic lymphocytic leukemia
CLP	Common lymphoid progenitor
CML	Chronic myelogenous leukemia
CMP	Common myeloid progenitor
CNTF	Ciliary neurotropic factor
ConA	ConcavalinA
CS	Cysteine to serine mutant
CSF	Colony stimulating factor
DA	Aspartic acid to alanine mutant
EAE	Experimental autoimmune encephalomyelitis
EGFR	Epidermal growth factor receptor
Egr1	Early growth response-1
Epo	Erythropoietin
ER	Endoplasmic reticulum
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FERM	Band 4.1/ezrin/radixin/moesin homology
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte colony stimulating factor
GH	Growth hormone
GM-CSF	Granulocyte-monocyte colony stimulating factor
GMP	Granulocyte/monocyte progenitor
GTG	Gold thioglucose

GVHD	Graft-versus-host disease
HDAC	Histone-deacetylase
HSC	Hematopoietic stem cell
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor associated kinase
IRF	Interferon-regulatory factor
IRS	Insulin receptor substrate
Jak	Janus kinase
JMML	Juvenile myelomonocytic leukemia
JNK	c-Jun N-terminal kinase
LIF	Leukemia inhibitory factor
LN	Lymph node
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEF	Murine embryonic fibroblast
MEP	Megakaryocyte/erythrocyte progenitor
MyD88	Myeloid differentiation antigen 88
NF-κB	Nuclear factor-κB
NO	Nitric oxide
OSM	Oncostatin M
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PI3K	Phosphoinositide 3-kinase
PIAS	Protein inhibitor of activated Stat
Prl	Prolactin
PTP	Protein tyrosine phosphatase

PTP-1B	Protein tyrosine phosphatase 1B
R	Receptor
RBC	Red blood cell
RLD	RING-finger-like zinc-binding domain
RTK	Receptor tyrosine kinase
SCF	Stem cell factor
SH2	Src-homology-2
SHP-1/2	SH2-containing phosphatase-1/2
SOCS	Suppressor of cytokine signalling
Stat	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
T-ALL	T-cell acute lymphoblastic leukemia
TC-PTP	T cell protein tyrosine phosphatase
TCR	T cell receptor
TEC	Thymic epithelial cell
TIR	Toll/IL-1 receptor
TIRAP	Toll/IL-1 receptor domain containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPO	Thrombopoietin
TRAF	TNF receptor associated factor
VHL	Von Hippel Lindau protein
XSCID	X-linked severe combined immunodeficiency
βc	Common β chain
γc	Common γ chain

7 ACKNOWLEDGEMENTS

First, I would like to thank my supervisors, Michel Tremblay and Wayne Lapp, for encouraging me to finish the work (in time) and for giving me my freedom. I am sure I was not always easy to live with, and I highly commend your patience.

Additional thanks to my thesis committee, Drs. Mark Featherstone, Philippe Gros, Marianna Newkirk, and Trevor Owens, for their critical comments and guidance.

I also wish to acknowledge the financial support received from the Canadian Institutes of Health Research, the Faculty of Graduate Studies and Research, McGill Cancer Centre, Canadian Society of Immunology and Défi Canderel.

I would also like to thank everyone who counted for something on the 11th floor: Frederick Nestel for letting me sit in his hot seat without making me cry, Rosmarie Siegrist-Johnstone for the long discussions, Christmas cookies and a pair of helping hands, as well as Enzo, Margot, Fiona and Gabrielle for their cheerful presence (and absence).

To make sure I will not leave anyone out: To all past an present members of the Tremblay lab, un gros merci! Special thoughts to Nadia and Annie with whom I experienced the efficiency and pleasure of team work; Melanie, Isabelle, Sofi and Jacinthe for the long lunch breaks; Emily for putting up with my failing memory that became worse with every pound I put on – thanks for the hat; Maxime for the long discussions on the secrets of western blotting and the stage show of Manowar; and Ailsa, Eva, and Janice for all the work they did for my (our?) mice making sure that no one, including me, mistreated them. I suppose it would have been possible for me to finish the work without you but it would have been nowhere near as enlightening or enjoyable.

Last but not least, special thanks to Jean-Sébastien for his help with the French abstract.

8 PUBLICATIONS AND CONTRIBUTION OF AUTHORS

First-author publications (included in thesis)

1. **Heinonen, K.M.**, Nestel, F.P., Newell, E.W., Charette, G., Seemayer, T.A., Tremblay, M.L., and Lapp, W.S. T Cell protein tyrosine phosphatase deletion results in fatal systemic inflammatory disease. 2004. Blood. 109:3457-64.
2. **Heinonen, K.M.**, Dubé, N., Bourdeau, A., Lapp, W.S., and Tremblay, M.L. Protein tyrosine phosphatase 1B negatively regulates macrophage development through CSF-1 signaling. 2006. Proc Natl Acad Sci. 103:2776-81.
3. **Heinonen, K.M.**, Bourdeau, A., Higgins, E.K., Loy, AL, and Tremblay, M.L. A complementary role for TC-PTP and PTP-1B in interferon-gamma signalling. In preparation.

Contributions of authors

1. FP Nestel and EW Newell provided figure 8b. G Charette assisted with figures 2 and 8a. TA Seemayer did the pathological analysis of sections depicted in figure 6.
2. N Dubé assisted with FACS experiments and had generated the retroviral vectors. A Bourdeau assisted with FACS experiments and the retroviral infection of macrophages.
3. A Bourdeau assisted with experiments on embryos. EK Higgins generated data for figures 4 and 5. AL Loy did all the genotyping of animals.

Other publications (not part of the thesis)

4. Dubé, N., Bourdeau, A., **Heinonen, K.M.**, Cheng, A., Lee Loy, A., and Tremblay, M.L. Genetic ablation of protein tyrosine phosphatase 1B accelerates lymphomagenesis of p53 null mice through the regulation of B cell development. 2005. Cancer Research. 65:10088-95.

   I assisted with FACS experiments and provided figure 5.

5. Bourdeau, A., Dubé, N., **Heinonen, K.M.**, Théberge, J.-F., Normandin, A., Lee Loy, A., and Tremblay, M.L. T Cell Protein Tyrosine Phosphatase (TC-PTP) is a critical regulator of bone marrow B cell development. In preparation.

   I assisted with FACS experiments.

Book chapters / reviews

6. Bourdeau, A., **Heinonen, K.M.**, Brunet, D.V., Tailor, P., Lapp, W.S., and Tremblay, M.L. Structure and function of the T-cell protein tyrosine phosphatase. 2004. Topics in Current Genetics. 5:185-200.

7. **Heinonen, K.M.** and Tremblay, M.L. Protein tyrosine phosphatase 1B in hematopoiesis. 2006. Cell Cycle. In press.

A Guy-Benoit et Mélianne,

pour m'avoir supportée et soutenue,

dans le meilleur et dans le pire.

Ja vanhemmilleni,

joiden vankkumaton usko minuun

vei minut läpi harmaan kiven.

1 CHAPTER I – INTRODUCTION

1.1 OVERVIEW OF THE THESIS

T cell protein tyrosine phosphatase (TC-PTP; also known as PTPN2) and protein tyrosine phosphatase 1B (PTP-1B; also known as PTPN1) are two closely related non-transmembrane phosphatases that have been implicated in the negative regulation of cytokine receptor signalling (reviewed in [1]). TC-PTP is ubiquitously expressed, but can be detected at higher levels in hematopoietic tissues than in most others. PTP-1B is also expressed throughout the system, but its function has been mostly characterized in tissues involved in glucose homeostasis, such as liver and muscle. Mice deficient in TC-PTP die perinatally from an ill-defined syndrome, characterized by various hematopoietic defects including anemia [2]. *In vitro* studies have also shown that the loss of TC-PTP in fibroblasts results in decreased proliferation and increased sensitivity to DNA damage [3]. On the other hand, *ptp1b*$^{-/-}$ mice were reported to be healthy and resistant to diet-induced obesity and diabetes [4]. This phenotype has resulted in several pharmaceutical companies attempting to inhibit PTP-1B in the hopes of alleviating Type II diabetes. Although PTP-1B was shown to regulate Jak2 phosphorylation in leptin [5] and growth hormone pathways [6] as well as interferon (IFN)-γ signalling in fibroblasts [7], no immune phenotype is apparent in *ptp1b*$^{-/-}$ mice. However, due to their high structural similarity, small-molecule inhibitors against PTP-1B also interfere with TC-PTP activity to a certain degree [8].

The aim of my thesis was therefore three-fold:

1) To better characterize the inflammatory syndrome in *tcptp*$^{-/-}$ mice and in particular, to determine the extent of hyperactive cytokine signalling;
2) To examine whether the increased activation of Jak2 signalling would lead to defective myeloid differentiation in the absence of PTP-1B; and
3) To determine whether TC-PTP and PTP-1B have a cooperative role *in vivo* and what the consequences of simultaneously inhibiting TC-PTP and PTP-1B would be by cross-breeding the two mutant strains.

This thesis has been divided into five sections. The first section is an overview of the literature as it pertains to cytokine signalling, the role of PTPs in its regulation, and the regulation of cytokine signalling in inflammation and myeloid development. The second section is based on a published manuscript that defines the role of TC-PTP as a regulator of inflammatory disease. The third section is based on a manuscript in revision that reports an essential role for PTP-1B in normal myeloid development through the regulation of colony stimulating factor (CSF)-1 signalling. The work presented in the fourth section examines the complementary role of TC-PTP and PTP-1B in monocyte/macrophage development and activation through the regulation of CSF-1 and IFN-γ signalling. The fifth section discusses the overall impact of TC-PTP and PTP-1B in hematopoiesis and proposes models to explain the similarities and differences in their function. It also includes a short discussion on how inhibiting TC-PTP and PTP-1B could be beneficial in specific situations.

1.2 CYTOKINE SIGNALLING

1.2.1 Cytokine receptors

1.2.1.1 Type I receptors

The traditional cytokine receptors are composed of transmembrane proteins with no intrinsic catalytic activity. They can be further divided into families based on the number and type of receptor subunits utilized as well as the structure of the individual ligands. Type I receptors all bind four-helix bundle cytokines [9], including various interleukins (IL), hormones, and colony stimulating factors. There is little sequence homology among the ligands, but their three-dimensional structure is highly conserved.

Some receptor subunits are shared by several ligands and used in conjunction with ligand-specific chains (see fig. 1.1 for the different receptor subfamilies). The common γ chain, for example, which is associated with X-linked severe combined immunodeficiency (XSCID) in humans [10, 11] is shared by IL-2, IL-7, IL-9, IL-15, IL-21 and IL-4, which explains the pleiotropic phenotype seen in XSCID. Other subfamilies can be defined as those using the common β chain (GM-CSF, IL-3 and IL-5) or gp130 (the IL-6 family). Yet other receptors are composed of homodimers of ligand-specific chains.

1.2.1.2 Type II receptors

The type II family is also known as the interferon receptor family and consists of the receptors for interferons as well as various interleukins, including IL-10 and IL-20. As a general structure, these receptors are formed by two chains, one of which has only a short cytoplasmic domain [12]. Once more, some of the receptor chains can be shared by several cytokines, and one ligand can also associate with different receptor complexes. The receptor chains also lack intrinsic catalytic activity and need to associate with cytoplasmic kinases for signaling.

Perhaps the prototype of type II receptors (see fig. 1.2 for receptor structure) is the IFN-γ receptor [13]. The ligand-binding α-chain (IFN-γR1) contains the long cytoplasmic domain necessary for the propagation of the signal. However, IFN-γR1 is not sufficient for signal transduction but requires association with the shorter β-chain (IFN-γR2). Both chains are known to bind cytoplasmic Janus kinases (Jaks) through their membrane-

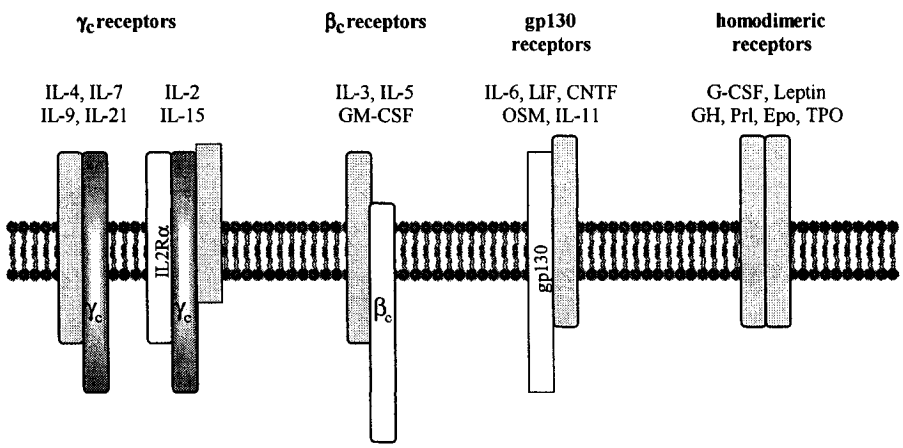


Figure 1.1 - Subfamilies of Type I cytokine receptors.

Common chains are identified and ligand-specific chains are depicted in solid grey. Abbreviations used: CNTF, ciliary neurotropic factor; LIF, leukemia inhibitory factor; OSM, oncostatin M; GH, growth hormone; Prl, prolactin; Epo, erythropoietin; TPO, thrombopoietin.

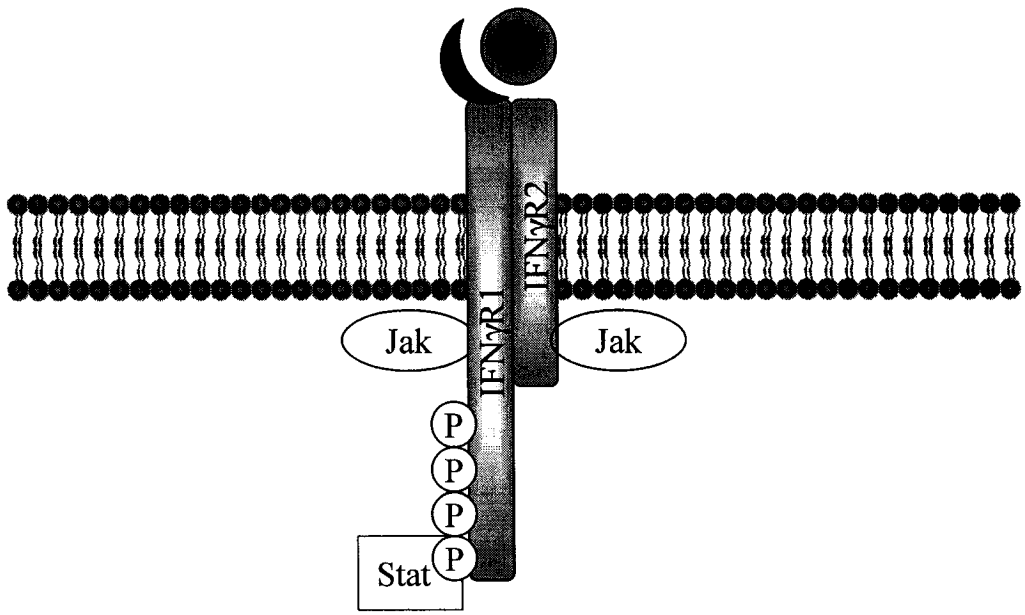


Figure 1.2 - Type II cytokine receptor structure.

Depicted is the IFN-γR with the ligand-binding and Stat-associating α chain (IFN-γR1) and the shorter ß chain (IFN-γR2). Adapted from [12].

proximal domains, whereas the more distal regions of the IFN-γR1 are necessary for the recruitment of signal transducer and activator of transcription (Stat)-1, the downstream signal transducer.

1.2.1.3 Receptor tyrosine kinases

Although the classical cytokine receptors initiate signalling through accessory molecules (Jak family kinases), there are several hematopoietic growth factors that associate with receptor tyrosine kinases. These include stem cell factor (SCF), colony-stimulating factor (CSF)-1 and Flt3 ligand. The three receptors, c-kit (CD117), CSF-1R (c-Fms; CD115) and Flt3 (CD135), respectively, belong to the Class III receptor tyrosine kinase family, which is generally characterized by five immunoglobulin (Ig)-like motifs in the extracellular domain and two intracellular kinase domains [14].

In the case of CSF-1R, the dimerization of the receptor results in the activation of the membrane-distal kinase and transphosphorylation of the receptor itself, which then serves as a recruitment site for cytoplasmic adaptors and signal transduction molecules [15]. In particular, phosphorylation of the activation loop tyrosine Y809 is necessary for macrophage proliferation and differentiation, while activation of the phosphoinositide 3-kinase (PI3K) pathway is crucial for CSF-1-dependent survival. CSF-1 and SCF signalling have also been shown to involve Stat5 [11, 15]. Some of the negative regulators of signaling are also shared between CSF-1R and the cytokine receptors.

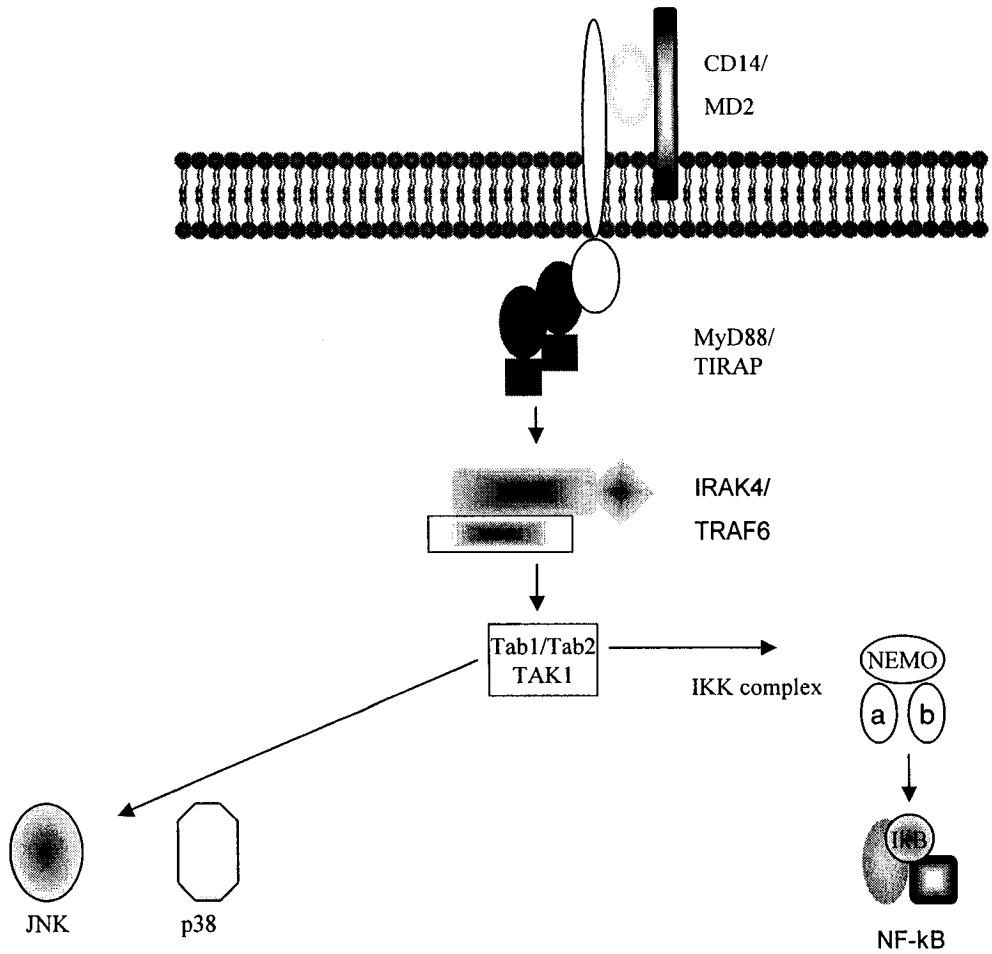


Figure 1.3 - Toll-like receptor signalling.

The Toll/IL-1R signalling pathway as exemplified by that of TLR4. Adapted from [16-17].

1.2.2 Toll/interleukin-1 receptors

1.2.2.1 Toll-like receptors (TLRs)

While B and T cells of the adaptive immune system recognize specific sequences of potentially pathogenic origin through their diverse and unique cell surface receptors, cells of the innate immune system have a more restricted repertoire of receptors that are able to recognize what are termed 'pathogen-associated molecular patterns' (PAMPs). These receptors are named after their *Drosophila* orthologue, Toll, and comprise eleven mammalian members (reviewed in [16, 17]). As a family, they are characterized by leucine-rich repeats in their extracellular domain and a conserved Toll/IL-1 receptor (TIR) domain in the cytoplasmic tail (see fig. 1.3). TIR domain associates with signalling molecules, including myeloid differentiation antigen 88 (MyD88) [18], IL-1 receptor associated kinase (IRAK) [19, 20], TNF receptor associated factor (TRAF)-6 [21], adaptor protein TIRAP [22, 23], mitogen-activated protein kinases (MAPK) and ultimately nuclear factor-κB (NF-κB).

Each TLR recognizes specific PAMPs. For example, bacterial lipopolysaccharide (LPS) binds to a complex containing TLR4 [24, 25], whereas other cell wall components, including peptidoglycan, associate with TLR2. Most TLRs are found on cell surface, but TLR9 is present on endosome membranes and therefore requires internalization of its ligand bacterial DNA as well as endosome maturation to become activated. Such intracellular localization correlates well with the nature of the ligand: TLR4 and TLR2 ligands are found on bacterial surface and require no processing, while that of TLR9 needs to be uncovered prior to receptor recognition. A recent report indicates that the intracellular localization of TLR9 may rather prevent unwanted receptor triggering by endogenous DNA [26].

In addition to binding to microbial products, TLRs also recognize endogenous ligands and therefore may play a role in autoimmunity and tissue damage. For example, TLR9 has been shown to bind to chromatin, which when found in complex with IgG2a is able to trigger rheumatoid factor release by auto-reactive B cells [27]. On the other hand, heat shock proteins released during tissue injury can be recognized by TLR4 and lead to an inflammatory response [28-30]. A summary of TLRs and their ligands can be found in table 1.1.

1.2.2.2 IL-1R and IL-18R

The receptors for IL-1 and IL-18 share the intracellular signalling pathway with TLRs and contain the same TIR motif in the cytoplasmic tail (reviewed in [31]). The extracellular domain consists of three Ig-like domains, which mediate ligand recognition. Both receptors are heterodimers of a ligand-binding chain (IL1Rα and IL18Rα) and an accessory receptor chain that does not associate directly with the ligand but is ligand-specific and required for high-affinity signalling. The IL-1 receptor family also includes ST2, which was recently found to bind IL-33 [32], and the orphan receptor single-immunoglobulin IL-1 related receptor (SIGIRR), which appears to play a role in negative regulation of Toll/IL-1 receptor signalling [33].

1.2.3 Positive control – JAK and STAT

1.2.3.1 General structure and function

The Janus kinases or Jaks are cytoplasmic tyrosine kinases that can be found constitutively associated with the cytokine receptors [34, 35]. There are four members to the family in humans and mice, Jak1, Jak2, Jak3 and Tyk2, with the same basal structure: an N-terminal band 4.1/ezrin/radixin/moesin homology (FERM) domain, followed by a Src-homology-2 (SH2)-domain, pseudokinase domain and a C-terminal kinase domain. The FERM domain mediates receptor association [36], whereas both the FERM [37] and the pseudokinase [38-40] domains contain sequences that regulate kinase activity. Upon receptor dimerization, Jaks become activated, presumably through autophosphorylation, and consequently phosphorylate the receptor allowing for the recruitment of Stats, which are also substrates for Jaks and mediate the downstream steps of cytokine signaling. Jak1, Jak2 and Tyk2 are ubiquitously expressed and used by both Type I and Type II cytokine receptors. In contrast, Jak3 associates exclusively with the common γ chain and its expression is lymphoid specific (for specific receptors, see table 1.2).

Stats are a family of transcription factors recruited to the cytokine receptors upon receptor phosphorylation. There are seven mammalian Stats, all of which are approximately 90kDa in size and contain the same structural features: a coiled-coil domain and a DNA-binding domain followed by a linker region, and SH2-domain and a

	Ligand	Refs.
TLR1*	Lipopeptides	[41, 42]
TLR2*	Lipoproteins (peptidoglycan, lipoteichoic acid, zymosan, lipoarabinomannan)	[43-46]
TLR3	dsRNA	[47, 48]
TLR4	LPS, heat shock proteins	[24, 25, 28]
TLR5	Flagellin	[49]
TLR6*	lipopeptides	[50]
TLR7	ssRNA, imidazoquinolines	[51, 52]
TLR8	ssRNA	[51]
TLR9	CpG DNA	[27, 53-55]
TLR10[$]	No known ligand	[56]
TLR11[$]	Profilin, uropathogenic bacteria	[57, 58]

Table 1.1 – Toll-like receptors and their ligands.

A summary of TLR ligands as identified mostly by gene deletions in mice. * TLR1 and TLR6 form heterodimers with TLR2. [$] There is no functional TLR10 in mouse and no functional TLR11 in human.

	Receptors	Knockout phenotype	Refs.
Jak1	γc cytokines (IL-2, IL-4, IL-7, IL-9, IL-15) gp130 cytokines (IL-6, IL-11, IL-12, CNTF, LIF, OSM) IFNs IL-10	Perinatally lethal due to failure to nurse; B and T lymphopoietic defects; Defective antiviral response	[59]
Jak2	βc cytokines (GM-CSF, IL-3, IL-5) gp130 cytokines cytokines with homodimeric receptors (GH, Prl, Epo, TPO, G-CSF, leptin) IFN-γ	Embryonic lethal due to defective erythropoiesis	[60, 61]
Jak3	γc cytokines	Defective lymphopoiesis; NK cell deficiency	[62, 63]
Tyk2	gp130 cytokines IFNα/β IL-10	Partial defect in antiviral response	[64, 65]

Table 1.2 – Jak usage by different cytokine receptor families.

Abbreviations used: CNTF, ciliary neurotropic factor; LIF, leukemia inhibitory factor; OSM, oncostatin M; GH, growth hormone; Prl, prolactin; Epo, erythropoietin; TPO, thrombopoietin. Adapted from [34].

transactivation domain. The SH2-domain mediates the initial docking to the receptor as well as dimerization of the phosphorylated Stats. Once phosphorylated by Jaks on a specific tyrosine residue within the transactivation domain, Stats form homo- or heterodimers and translocate to the nucleus to activate target gene transcription. There is no classical nuclear localization signal on Stats, but nuclear entry appears dependent on dimerization as well as signals within the DNA-binding domain. Stat3 and Stat5 are the most widely used Stats, while Stat1 is mostly restricted to the interferon receptors (summarized in table 1.3). Phenotypes of Jak and Stat –deficient mice are summarized in tables 1.2 and 1.3, whereas their association with human disease is discussed below.

1.2.3.2 Jak2

Jak2 deletion in mice is lethal in embryonic development [60, 61] due to a loss of erythropoietin signaling and thereby a blockage in definitive erythropoiesis. Other cytokines affected by the loss of Jak2 are common β family members IL-3, IL-5 and GM-CSF [61] as well as thrombopoietin and IFN-γ [60, 61]. In humans, Jak2 has been implicated in leukemogenesis through the TEL-Jak2 fusion chimeras. The fusion results in a constitutively active Jak2 dimer in children with T-cell acute lymphoblastic leukemia (T-ALL) [66]. Similar translocations have also been observed in early pre-B acute lymphoid leukemia and atypical chronic myelogenous leukemia (CML) [67]. Further studies have shown that ectopic expression of the TEL-Jak2 fusion protein results in growth factor independence and constitutive Stat activation *in vitro* [68, 69] and in fatal lympho- and myeloproliferative syndrome in bone marrow transplanted mice [68]. A gain-of-function mutation in Jak2 has been recently associated with increased incidence in chronic myeloproliferative syndrome [70-72] AML and CML [73]. These studies demonstrate that, in addition to being indispensable for normal erythropoiesis, Jak2 is also critical in the regulation of myelopoiesis, at least through IL-3.

1.2.3.3 Jak3

In contrast to other Jaks, Jak3 expression is restricted to the hematopoietic compartment and is inducible upon activation [74-76]. It interacts with the γc chain and is indispensable for signaling by the γc receptors, including IL-2 and IL-7. Jak3 deficiency in humans results in autosomal recessive SCID characterized by a lack of T cells and NK

	Receptors	Knockout phenotype	Refs.
Stat1	IFNs	Defective antiviral response; Defective macrophage activation	[77, 78]
Stat2	IFNα/β	Defective antiviral response	[79]
Stat3	IL-6 family (IL-6, IL-11, CNTF, LIF, OSM) G-CSF, leptin, GH, IL-10	Embryonic lethal; Defective acute phase response (hepatocyte-specific deletion); Defective anti-inflammatory control of macrophage activation (macrophage-specific deletion); Impaired thymic function (TEC-specific deletion); Impaired wound healing (keratinocyte-specific deletion); Defective mammary gland involution (mammary gland-specific deletion)	[80-85]
Stat4	IL-12	Impaired Th1 differentiation; Impaired NK cell cytotoxicity	[86, 87]
Stat5a Stat5b	γc cytokines (IL-2, IL-7, IL-9, IL-15) βc cytokines (GM-CSF, IL-3, IL-5) GH, Prl, Epo, TPO	Defective mammary gland development (Stat5a); Defective GM-CSF response (Stat5a); Loss of sexually dimorphic growth (Stat5b); Impaired IL-2 response (Stat5a/b); Foetal anaemia (Stat5a/b); Decreased hematopoietic progenitor function (Stat5a/b); Diminished GM-CSF response (Stat5a/b); Decreased IL-7 response (Stat5a/b)	[88-96]
Stat6	IL-4, IL-13	Impaired Th2 differentiation	[97-101]

Table 1.3 – Stat usage by different cytokine receptor families.

Abbreviations used: CNTF, ciliary neurotropic factor; LIF, leukemia inhibitory factor; OSM, oncostatin M; GH, growth hormone; Prl, prolactin; Epo, erythropoietin; TPO, thrombopoietin; TEC, thymic epithelial cell. Adapted from [34, 102].

cells but the presence of B lymphocytes [103-105]. Interestingly, in mice Jak3 signalling is required for both T and B lymphopoiesis [62, 63]. Similarly, IL-7 signaling is necessary for B cell development in mice, as *IL7R*$^{-/-}$ mice lack functional B cells [106], but humans with mutations in IL-7R develop T cell deficient, B cell sufficient SCID [107]. NK cell deficiency in both humans with SCID and *jak3*$^{-/-}$ mice stems from the loss of IL-15 signaling. Mice deficient in Jak3 also display age-dependent expansion of the myeloid compartment, presumably through stimulation by activated residual T lymphocytes rather than a direct effect of Jak3 on a particular cytokine pathway [108].

1.2.3.4 Stat1

The first Stat to be deleted in mice was Stat1. Despite the fact the Stat1 has been reported to be activated in the context of a variety of different cytokines, the main phenotype detected in Stat1 deficient mice was an impaired response to interferons [77, 78]. The impaired induction of interferon response factors resulted in increased susceptibility to viral infections [77, 78] as well as to *Listeria monocytogenes* [78], whose clearance is dependent on normal macrophage activation by interferons. There was no detectable change in hematopoiesis [77], excluding the possibility that defective development would affect the antiviral response. Signalling downstream of other receptors known to activate Stat1 was not impaired [78].

The decreased sensitivity to IFN also rendered Stat1-deficient mice more susceptible to the development of tumours, either through the deletion of p53 or by treatment with methylcholanthrene [109]. This was shown to be due to the loss of Stat1 in the tumour itself and not due to decreased immune surveillance. One potential mechanism lies in the Stat1-independent functions of interferons: in the absence of Stat1, stimulation with interferon resulted in activation of *c-myc*, which is a known proto-oncogene and actually suppressed by activated Stat1 in a manner which was dependent on the phosphorylation of Stat1 on S727 [110].

1.2.3.5 Stat3

Tissue-specific targeting of Stat3 has revealed a variety of immune and non-immune phenotypes (table 1.3), and Stat3 therefore has both antiapoptotic (in the thymus) and proapoptotic (in mammary gland) properties, depending on the physiological setting.

Stat3 activity appears to correlate with increased oncogenicity, though. Overexpression of a constitutively dimerized (and presumably active) form of Stat3 resulted in transformation of immortalized fibroblasts as indicated by increased colony formation in soft agar as well as tumour formation in nude mice [111]. Enhanced or constitutive activation of Stat3 is also associated with a variety of hematopoietic malignancies, including multiple myeloma, Burkitt's lymphoma, acute myeloblastic leukemia (AML), and chronic lymphocytic leukemia (CLL) (reviewed in [102]), as well as solid tumours such as breast cancer and ovarian carcinoma.

1.2.3.6 Stat5a and Stat5b

Stat5a and Stat5b are highly homologous (>90%) but encoded by two different genes. Both proteins are activated by a variety of cytokines (see table 1.3), but appear to have certain distinct functions. As Stat5 is significantly involved in cytokine-mediated proliferation and survival, it can be expected to also play a role in oncogenesis. Constitutive Stat5 activation has indeed been reported in AML, TEL-Jak2 positive acute lymphoblastic leukemia (ALL), as well as Bcr-Abl or TEL-PDGFβR positive CML (reviewed in [102]). Interestingly enough, exogenous expression of TEL-Jak2 in Stat5a/b deficient cells [112] did not result in myeloproliferative disease, a defect which could be rescued by expressing either Stat5a or oncostatin M, a Stat5 target gene. Conversely, Stat5a/b deficiency did not prevent transformation by v-Abl or Bcr-Abl [96], suggesting that, depending on the physiological context, Stat5 activation in oncogenesis can be either a primary or secondary event.

1.2.4 Negative control – SOCS, PIAS and PTPs

1.2.4.1 Protein Tyrosine Phosphatases

Both Jaks and Stats become phosphorylated on tyrosine residues during the activation of a cytokine signal. The role of protein tyrosine phosphatases (PTPs) is to dephosphorylate the activated Jaks and Stats, thereby downregulating a once activated response. The family of human PTPs (reviewed in [113, 114]) consists of 38 classical PTPs (i.e. acting solely on tyrosines), 61 dual-specific phosphatases (i.e. those dephosphorylating serine and/or threonine in addition to tyrosine) as well as 8 phosphatases that do not belong to either major class. The vast majority of human PTPs have murine orthologues. For the purpose of this thesis, the term PTP refers only to classical PTPs, which are depicted in figure 1.4.

The catalytic activity of PTPs resides in the highly conserved signature motif with crucial cysteine and arginine residues (Cx_5R) [115, 116]. The active site cysteine is of particular importance, and a cysteine → serine mutant PTP (PTP CS) will still bind to its substrate but is no longer able to dephosphorylate the phosphotyrosine. This mutation increases the stability of the enzyme-substrate interaction and is often referred to as the "substrate-trapping" mutant [117]. Another such mutation involves an aspartic acid residue that serves as the general acid during dephosphorylation. Mutation of this amino acid to an alanine (PTP DA) also increases the stability of the enzyme-substrate complex and allows for better co-precipitation. Both PTP CS and PTP DA mutants are widely used for identification of PTP substrates.

Most PTPs are expressed in hematopoietic cells and in certain cases their deletion results in immune phenotypes in mice (reviewed in [118]). Only some of these are associated with Jak/Stat signalling, though. The first phosphatase shown to regulate Jak phosphorylation was SH2-containing PTP-1 (SHP-1) [119]. Since then, other PTPs, cytoplasmic, nuclear and trans-membrane, have been shown to participate in the control of cytokine signalling in stimulus- and tissue-specific fashion. These are summarized in Table 1.4 and discussed in greater detail below.

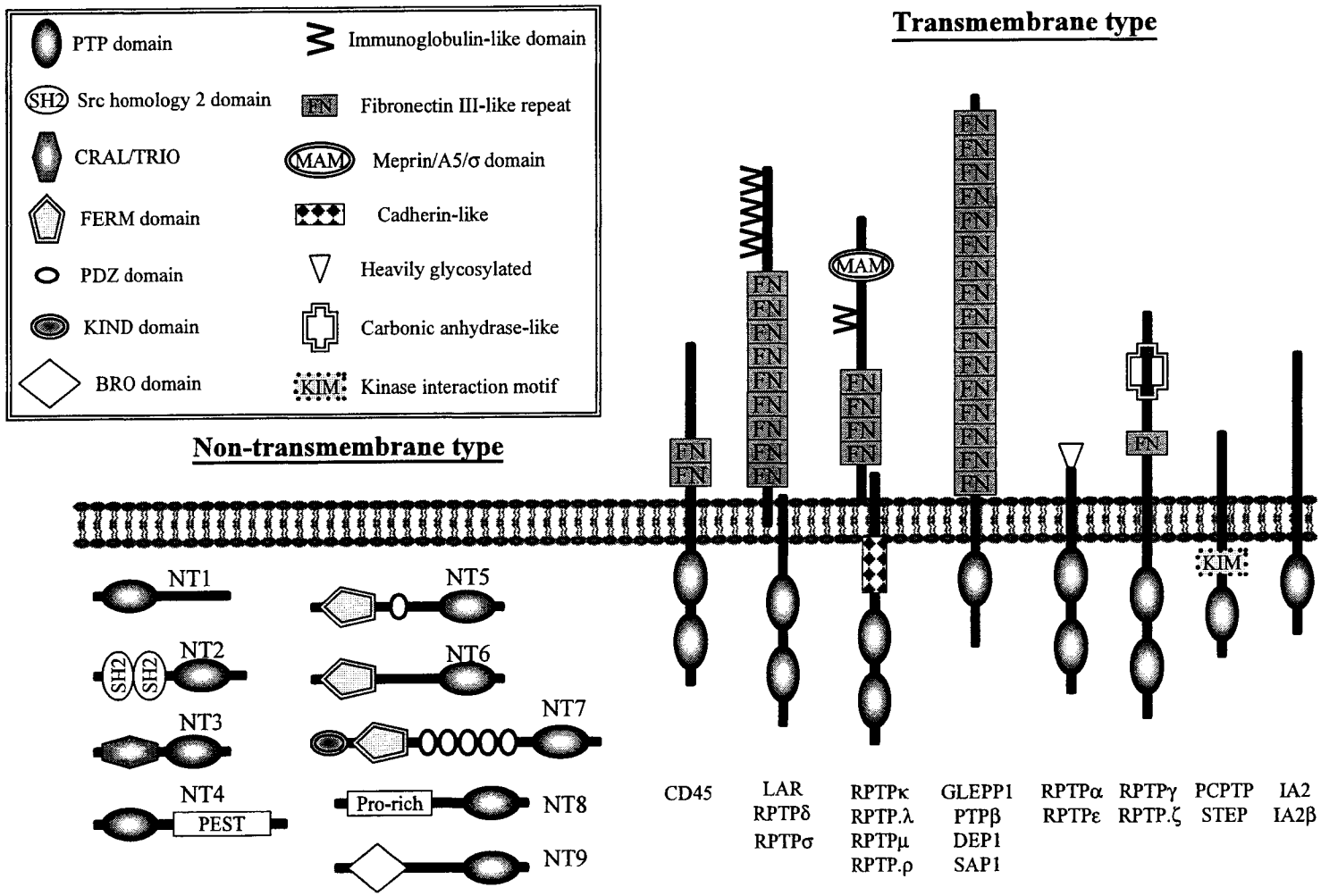
	Substrate	Cytokine	Refs.
CD45 (PTPRC)	Jak1, Jak2, Jak3, Tyk2	IL-3, SCF, Epo, IFNα	[120]
	?	IL-2	[121]
	?	IL-7	[122]
RPTP-ε (PTPRE)	Stat3	IL-6, LIF	[123]
		IL-6, IL-10	[124]
PTP-1B (PTPN1)	Jak2	Leptin	[5, 125]
		GH	[6]
	Jak2, Tyk2	IFN-γ, IFN-α/β	[7]
	Stat5	Prl	[126]
	EpoR	Epo	[127]
TC-PTP (PTPN2)	Stat1	IFNγ	[128]
	Stat3	IL-6	[128, 129]
	Stat5	Prl	[130]
	Jak1, Jak3	IFN-γ, IL-2	[131]
SHP-1 (PTPN6)	EpoR / Jak2	Epo	[119]
	IFNα/βR, Jak1, Stat1	IFNα/β	[132]
	Jak2	GH	[133]
	Stat6	IL-4, IL-13	[134]
	IL-3R	IL-3	[135]
SHP-2 (PTPN11)	gp130	IL-6	[136]
	Stat1	IFN-γ	[137]
	?	IFN-α	[138]

Table 1.4 – Protein tyrosine phosphatases in cytokine signalling.

Summary of the phosphatases and the pathways they affect. Abbreviations used: SCF, stem cell factor; Prl, prolactin; GH, growth hormone; Epo, erythropoietin; LIF, leukemia inhibitory factor.

Figure 1.4 – Classical protein tyrosine phosphatases.

Structures of the main groups of classical PTPs. Adapted from [113-114]. Non-transmembrane groups : NT1, TC-PTP and PTP-1B; NT2, SHP-1 and SHP-2; NT3, PTP-MEG2; NT4, PTP-PEST, LyPTP and PTP-HSCF; NT5, PTPH1 and PTP-MEG1; NT6, PTPD1 and PEZ; NT7, PTP-BAS; NT8, Typ-PTP; NT9, HD-PTP. Abbreviations used: CRAL/TRIO, cellular retinaldehyde binding protein/trio homology (Sec14p homology); FERM, band 4.1/ezrin/radixin/moesin homology; PDZ, postsynaptic density-95/discs large/ZO1 homology; KIND, kinase N lobe-like domain; BRO, baculovirus BRO homology.


PTP domain
Src homology 2 domain
CRAL/TRIO
FERM domain
PDZ domain
KIND domain
BRO domain
Immunoglobulin-like domain
Fibronectin III-like repeat
Meprin/A5/σ domain
Cadherin-like
Heavily glycosylated
Carbonic anhydrase-like
Kinase interaction motif
Transmembrane type
Non-transmembrane type
NT1
NT2
NT3
NT4
PEST
NT5
NT6
NT7
Pro-rich
NT8
NT9
CD45
LAR
RPTPδ
RPTPσ
RPTPκ
RPTP.λ
RPTPμ
RPTP.ρ
GLEPP1
PTPβ
DEP1
SAP1
RPTPα
RPTPε
RPTPγ
RPTP.ζ
PCPTP
STEP
IA2
IA2β

1.2.4.2 SOCS

Suppressors of cytokine signalling (SOCS) are a group of proteins whose expression is induced in response to cytokine stimuli and whose function is to downregulate the said signal, in the manner of a classical negative feedback inhibitor. There are eight members to the mammalian SOCS family (reviewed in [139-141]): SOCS-1 through –7 and cytokine-induced SH2 protein (CIS). They contain an SH2 domain, through which they presumably bind to their target proteins as well as a C terminal SOCS box domain and a largely variable N terminal domain. The SOCS box is also found in other proteins, including the tumor suppressor Von Hippel Lindau (VHL) protein, in which it is known to mediate association with a E3 protein ubiquitin ligase complex [142, 143]. Indeed, binding of SOCS has also been reported to increase degradation of its target proteins [144-146]. In addition to targeting its substrates for ubiquitination, SOCS family proteins can also inhibit cytokine signalling by competing for binding sites on the receptor [147-149] or by direct binding to Jaks [148-150] (see figure 1.5). The phenotypes of mice with genetically modified SOCS loci are summarized in table 1.5.

1.2.4.3 PIAS

Mammalian protein inhibitors of activated Stat (PIAS) comprise four members, PIAS1, PIAS3, PIASx, and PIASy, the last three of which have two splice variants (reviewed in [151]). They have an amino-terminal SAP domain, which mediates corepressor recruitment [152], a RING-finger-like zinc-binding domain (RLD) in the central portion, and an acidic and a serine/threonine-rich domain in the carboxy-terminal end. The RLD domain is required for the small ubiquitin-like modifier (SUMO) E3 ligase activity of PIAS proteins [153]. In addition to recruiting corepressors and targeting Stats for degradation through SUMOylation, PIAS can also directly interfere with DNA-binding activity [154]. The different mechanisms are depicted in figure 1.5. In addition to inhibiting Stat-mediated signalling, PIAS can also associate and interfere with other transcription factors, including interferon-regulatory factors (IRF), NF-κB p65, SMADs and p53 [151]. Pathways targeted by individual PIAS are summarized in table 1.5.

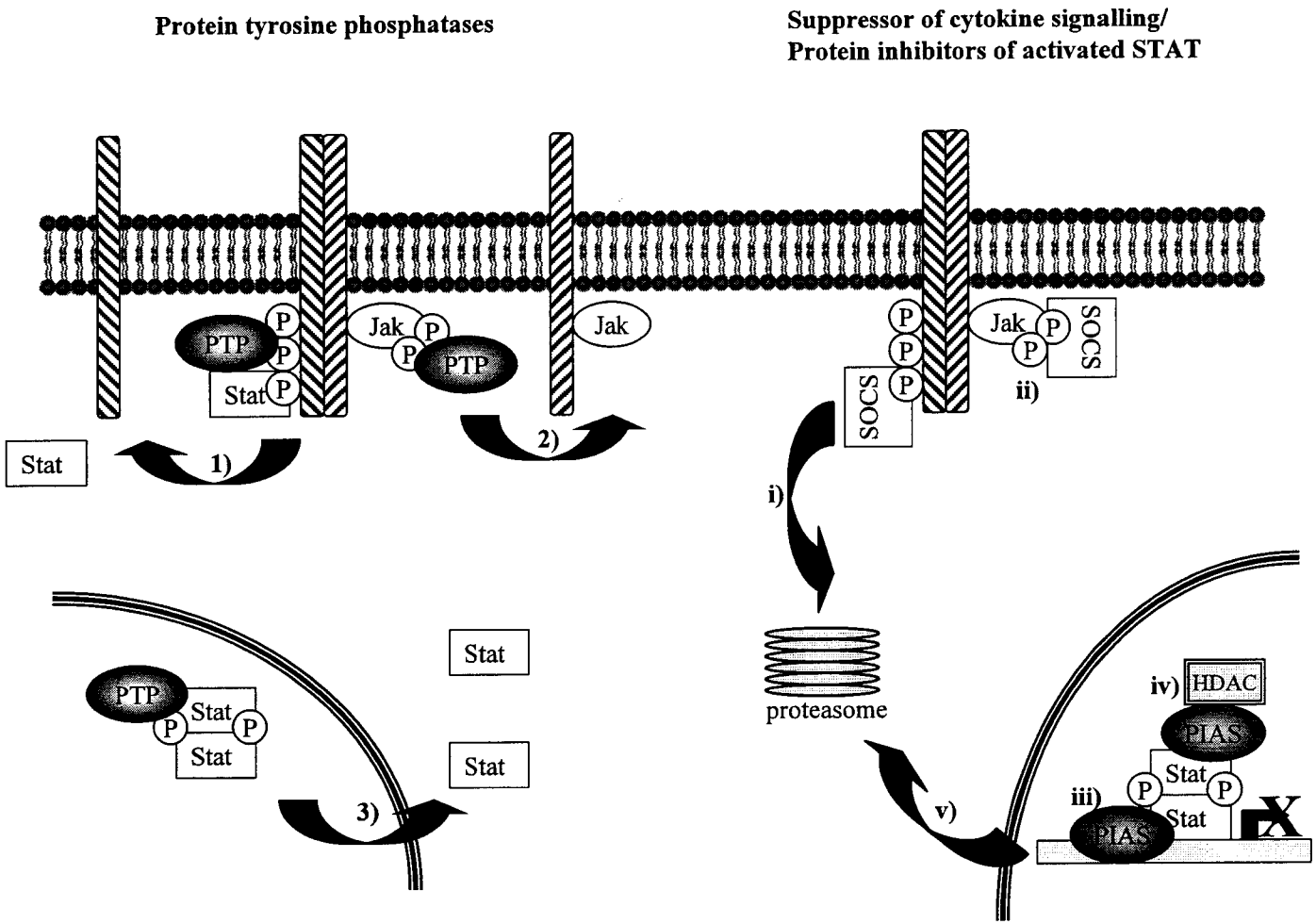
	Pathways targeted/ Mechanism	Phenotype of mice	Refs.
CIS	GH, IL-2, IL-3, IL-15; Receptor association	cis(tg): growth retardation; decreased NK(T) development	[147, 155-157]
SOCS1	IFN-γ (major); Jak2 binding (pseudosubstrate), Jak degradation	socs1$^{-/-}$: Liver degeneration; lymphopenia; massive inflammation; TLR hypersensitivity	[146, 150, 158-161]
SOCS2	GH, IGF-1; Receptor association	socs2$^{-/-}$: gigantism	[162, 163]
SOCS3	IL-6, LIF, G-CSF	*socs3*$^{-/-}$: embryonic lethality (placental defects); neutrophilia (*socs3*$^{-/-}$*lif*$^{-/-}$); auto-immune/inflammatory disease (hematopoietic specific deletion)	[164-168]
SOCS4	EGFR	N/A	[169]
SOCS5	IL-4? EGFR	socs5$^{-/-}$: no hematopoietic defects detected	[169-172]
SOCS6	c-kit, IRS-4	socs6$^{-/-}$: mild growth retardation	[173, 174]
SOCS7	unknown	socs7$^{-/-}$: hydrocephalus	[175]
PIAS1	IFNs (Stat1); Inhibit DNA binding	*pias1*$^{-/-}$: hypersensitivity to LPS, increased resistance to viral and bacterial infections	[176-178]
PIAS3	Stat3; Inhibit DNA binding	*N.A.*	[154]
PIASx	Stat4; Recruitment of HDAC	*N.A.*	[179]
PIASy	Stat1	*piasy*$^{-/-}$: no defect in interferon signalling	[180, 181]

Table 1.5 – Ligand-specificity of SOCS and PIAS.

Summary of pathways targeted by individual SOCS and PIAS and the phenotypes of gene-targeted mice.

Figure 1.5 – Mechanisms of negative regulation of cytokine signalling.

A) PTPs dephosphorylate 1) activated receptors, thus preventing recruitment of downstream accessory molecules, including Stats; 2) activated Jaks, thus deactivating them and decreasing phosphorylation of substrates, including receptor chains and Stats; or 3) phosphorylated Stats, thus resulting in their disassociation and exit from the nucleus. ***B)*** i) SOCS2 and CIS associate with receptor chains, thereby preventing recruitment of downstream accessory molecules and promoting the ubiquitination and degradation of the receptor; ii) SOCS1 and SOCS3 can associate directly with Jaks, thus preventing phosphorylation of substrates and promoting the ubiquitination and degradation of Jaks; iii) PIAS1 and PIAS3 can prevent the binding of target Stats to DNA; iv) PIASx and PIASy can inhibit Stat-dependent transcription through the recruitment of corepressors, including HDACs; v) PIAS promote sumoylation of target Stats and hence their degradation.


Protein tyrosine phosphatases
Suppressor of cytokine signalling/
Protein inhibitors of activated STAT
PTP
P
Stat
Jak
1)
2)
SOCS
i)
ii)
proteasome
iii)
iv)
HDAC
PIAS
v)
3)

1.3 PTPs IN CYTOKINE SIGNALING

1.3.1 Transmembrane type

1.3.1.1 CD45 (PTPRC)

CD45 is expressed on all nucleated hematopoietic cells and was the first transmembrane or receptor-type tyrosine phosphatase to be identified in 1988 [182]. It has since been shown to play an important role in regulating the signalling threshold of the T and B cell receptors (TCR and BCR) for antigen (reviewed in [183]). The main mechanism for the regulation of TCR and BCR signalling by CD45 is through its ability to modulate the activity of Src family kinases, which are the primary kinases to become activated after antigen stimulation. Initially CD45 was thought to activate Src kinases through dephosphorylation of an inhibitory tyrosine residue, which was consistent with the increased signalling threshold detected in *CD45*$^{-/-}$ cells [184]. However, loss of CD45 in macrophages results in increased adherence [185] due to increased Hck activity at focal adhesion complexes, suggesting that CD45 can also negatively regulate Src activity, at least in the context of integrin signalling. A possible explanation for the seemingly opposite functions of CD45 lies in its localization with respect to the signalling complex: TCR and BCR are recruited to lipid rafts upon activation, and CD45 is excluded from such rafts and thus cannot participate in the negative regulation of Src kinases. Conversely, integrin signalling complexes may include CD45 [183].

In addition to regulating Src kinases, CD45 has also been suggested to play a role in cytokine signalling [120]. It associates with Jaks *in vitro* and dephosphorylates and inactivates them. The association is through the membrane-distal PTP domain of CD45, which is catalytically inactive. Co-expression studies showed that CD45 can negatively regulate IL-3 and SCF –mediated proliferation and colony formation, Epo-dependent erythropoiesis and the interferon response [120]. Recently, another group reported sustained and heightened Jak1 and Stat5 phosphorylation in IL-7-dependent cell lines from *CD45*$^{-/-}$ mice [122]. Although they detected increased Src kinase activity, which could also increase Jak/Stat phosphorylation, Irie-Sasaki et al. showed that Src inhibitors had no effect on Jak hyperphosphorylation in response to IL-3 in *CD45*$^{-/-}$ cells [120]. It

does, therefore, appear that CD45 is a *bona fide* Jak phosphatase, although further studies would help to clarify the extent of its contribution to the control of cytokine signalling.

1.3.1.2 RPTP-ε (PTPRE)

Receptor protein tyrosine phosphatase-epsilon (RPTP-ε; also known as PTPRE) [186] exists in four forms resulting from transcription from two separate promoters, proteolytic cleavage and translational initiation from internal sites. The full length form is membrane-spanning and has been found increased in mouse mammary tumors [187], where it contributes to the activation of Src family kinases. The cytoplasmic form created by alternate promoter usage is primarily expressed in monocyte-macrophage lineage cells [188, 189], and has been shown to inhibit IL-6, LIF and IL-10 –mediated signalling [123, 124] in overexpression studies. *In vivo* evidence for a role of RPTP-ε in cytokine signalling is still lacking: mice deficient in RPTP-ε show nervous defects [190] and deficient osteoclast maturation presumably due to defects in cytoskeletal arrangement [191] but no immune phenotype has been reported.

1.3.2 Non-transmembrane type

1.3.2.1 SHP-1 (PTPN6)

SH2 domain-containing phosphatase –1 (SHP-1) is a primarily hematopoietic phosphatase [192] with two N-terminal SH2 domains, one of which plays a negative regulatory role through binding to the catalytic domain and thereby preventing substrate binding [193]. It was found to be mutated in *motheaten* (*me/me*) mice [194, 195], which are characterized by multiple hematopoietic defects, leading to chronic inflammation and systemic autoimmune disease. This phenotype clearly indicated that SHP-1 was able to regulate immune cell development and function at several levels.

SHP-1 was also the first phosphatase reported to regulate Jak/Stat-mediated signals [119]. It has been shown to associate with the EpoR and dephosphorylate Jak2 [119], negatively regulate Stat6-mediated signalling in the IL-4 and IL-13 pathways [134], associate with the IFNα/β receptor to decrease Jak1 and Stat1 phosphorylation [132], and downregulate growth hormone receptor signals through Jak2 [133]. It also binds to and dephosphorylates the IL-3R [135].

Activation of Stats is associated with transformation, and one possible mechanism for increased Stat phosphorylation is through decreased inhibitory phosphatase activity. Decreased levels of SHP-1 due to hypermethylation of the locus have been detected in acute myeloid leukemia and multiple myeloma cells [196, 197]. However, hypermethylation had no prognostic value in either case. Decreased SHP-1 expression and concomitant Stat3 hyperphosphorylation was also reported in adult T-cell leukemia [198]. Furthermore, when primary cells were infected with human T cell leukemia virus, the gradual loss of SHP-1 correlated with growth factor (IL-2) independence and cellular transformation. In yet another study, the loss of SHP-1 expression correlated with progression of myelodysplastic syndrome to acute leukemia [199]. Downregulating SHP-1 activity therefore seems to be associated with haematological malignancies, even if there may not always be any causative relationship.

SHP-1 is also known to dephosphorylate receptor tyrosine kinases, including colony stimulating factor 1 (CSF1) receptor, the major receptor for macrophage differentiation and proliferation [200]. *me/me* macrophages show an increased proliferation rate in response to CSF-1 and prolonged growth factor-independent survival. This was shown to be associated with increased p62(DOK) phosphorylation [201]. Accordingly, SHP-1 deficiency resulted also in increased osteoclast-formation and decreased bone density [202, 203]. In addition to proliferation, SHP-1 regulates macrophage function through modulation of FcγR-mediated phagocytosis [204], and is the target protein through which *Leishmania donovani* modulates macrophage activity and escapes degradation [205, 206]. This is presumably due to increased SHP-1 activity and corresponding decreased signal transduction in response to IFN-γ [207, 208].

1.3.2.2 SHP-2 (PTPN11)

SHP-2 is structurally similar to SHP-1 and is recruited to receptor tyrosine kinases (RTKs), but rather than deactivating RTK signalling, it appears to promote signal transduction through dephosphorylation of negative regulators [209, 210] and activation of downstream signalling cascades, such as the PI3K pathway [211]. It can be activated by phosphorylation [212, 213], and this C-terminal modification is required for relaying signals downstream of certain receptors but not others [214]. In addition, SHP-2 can also

play a negative regulatory role in certain cytokine signalling pathways, notably IL-6 [136], IFN-γ [137] and IFN-α [138].

Mice deficient in SHP-2 die early in embryogenesis and present defective gastrulation [215, 216]. This correlates with defective fibroblast growth factor responses. Conversely, activating mutations in SHP-2 are associated with Noonan syndrome, a developmental disorder characterized by shortened stature, facial abnormalities and cardiac defects [217, 218]. Noonan syndrome is associated with an increased incidence in juvenile myelomonocytic leukemia (JMML); sporadic SHP-2 mutations (known gain-of-function and unknown mutations) are detected in JMML but also in myelodysplastic syndrome, B-ALL, AML and neuroblastoma [219, 220]. In brief, gain-of-function and potentially other mutations in SHP-2 correlate with tumorigenicity and make SHP-2 an interesting target for anti-cancer therapy.

1.4 TC-PTP AND PTP-1B

1.4.1 Substrate specificities

The work presented in subsequent chapters concentrates on the role of TC-PTP and PTP-1B in the regulation of macrophage development, activation and function, which is why these two phosphatases are discussed separately and in greater detail here.

1.4.1.1 T cell protein tyrosine phosphatase

TC-PTP was originally cloned in human T cells [221] but is expressed ubiquitously in mice, although at higher levels in hematopoietic tissues [222]. There are two isoforms, the initially cloned 48-kDa form, which localizes to the endoplasmic reticulum [223], and the predominant 45-kDa form, which lacks the C-terminal tail that would target it to the ER but rather contains a bipartite nuclear localization sequence [224, 225]. The 45kDa form is also the only one detectable in mice. In rat, by contrast, four different splice variants exist [226]. For the purpose of the thesis, TC-PTP refers to the 45-kDa form (mouse or human), and TC48 to the human 48-kDa form.

Although TC-PTP is mostly found in the nucleus, only a few of its proposed substrates are nuclear. The first published TC-PTP substrates were the epidermal growth factor receptor (EGFR) and the adaptor protein p52shc [227]. The association with EGFR

did not affect downstream signalling through the p42/p44 MAPK pathway, but did inhibit recruitment of Grb2, another adaptor molecule, to the EGFR/Shc complex. The same authors also reported that EGFR-mediated PI3K and c-Jun N-terminal kinase (JNK) activation was inhibited by TC-PTP [228], but there was no direct association. To explain how a nuclear enzyme can have a membrane-targeted substrate, Tiganis et al. [227] observed that TC-PTP was able to exit the nucleus upon stimulus and bind EGFR in the cytoplasm. This was later reported to occur during cellular stress and to be independent of common nuclear export mechanisms [229] but rather operate by passive diffusion. A recent report defines a link between integrin signalling, EGFR signalling and TC-PTP by demonstrating that $\alpha_1\beta_1$-integrins activate TC-PTP and recruit it to the membrane, where it would downregulate EGFR signalling [230].

In addition to EGFR, a truncated form of EGFR [231] the platelet-derived growth factor receptor (PDGFR) [3, 232] and the insulin receptor [233, 234] are other RTKs whose signalling is negatively regulated by TC-PTP. However, *in vivo* evidence for a physiological role of TC-PTP in RTK signalling is still lacking.

Considering the largely hematopoietic phenotype of the *tc-ptp*$^{-/-}$ mouse [2] as well as the tissue distribution of the enzyme, it was perhaps not surprising that TC-PTP was found to modulate cytokine signalling. Stat5 [130], Stat3 [128, 129] and Stat1 [128] have been shown to be dephosphorylated by TC-PTP in response to prolactin, IL-6 and IFN-γ, respectively. Jak1 and Jak3 have also been identified as substrates for TC-PTP [131] downstream of IFN-γR and IL-2R, respectively. In addition, TC-PTP has been reported to bind to the phosphorylated growth hormone receptor [235], although the physiological importance of such association is yet unclear.

1.4.1.2 Protein tyrosine phosphatase 1B

PTP-1B is a ubiquitously expressed cytosolic phosphatase that is localized to the endoplasmic reticulum [236]. It was the first PTP whose crystal structure was resolved [237], and has since become the prototypic non-transmembrane PTP. It has an N-terminal catalytic domain, followed by two proline-rich domains and a hydrophobic tail that targets it to the cytoplasmic face of the ER. Its phosphorylation accompanies cell cycle progression [238], and has been proposed to regulate its catalytic activity. It can also be

cleaved by calpain during platelet aggregation, and this cleavage results in its release from the ER membrane to the cytoplasm [239].

Similar to TC-PTP, the subcellular localization of PTP-1B is in conflict with the localization of its reported substrates. PTP-1B regulates several RTKs, including EGFR [240, 241], PDGFR [241-243], insulin receptor [4, 244, 245] and insulin-like growth factor type I receptor [246, 247]. More recently, PTP-1B has been shown to regulate ER stress responses [248], but thus far no ER-localized PTP-1B substrates have been identified.

In addition to RTKs, PTP-1B has other membrane-associated substrates. It has been shown to dephosphorylate Src on the inhibitory tyrosine residue, thus positively regulating its activity [249] in integrin-dependent signaling. More recently, focal adhesion kinase (FAK) and α-actinin were also identified as substrates for PTP-1B downstream of integrins [250]. The adaptor protein p130cas [251] can associate with PTP-1B through one of the two proline-rich domains in the C-terminus of PTP-1B, forming another link between PTP-1B and integrin signaling. PTP-1B can also dephosphorylate the negative regulator p62dok [243] and thus positively influence Ras signaling. The oncoprotein p210 Bcr-abl, which is found in CML, is another substrate of PTP-1B, and certain CML-derived cell lines overexpress PTP-1B [252]. Lastly, PTP-1B can regulate cytokine signaling through dephosphorylation of Jak2 and Tyk2 downstream of leptin [5, 125], growth hormone [6], IFN-γ and IFN-α/β [7], Stat5a/b downstream of prolactin [126] as well as through a direct association with the Epo receptor [127].

1.4.2 Knockout mice

1.4.2.1 T cell protein tyrosine phosphatase (tcptp$^{-/-}$)

TC-PTP-deficient mice were generated in our laboratory and have a mutant allele containing a deletion of the catalytic domain which results in the loss of expression of the gene product [2]. The *tcptp*$^{-/-}$ mice are born at the expected Mendelian ratio and appear physically normal until 10-14 days of age, although some signs of growth retardation can be detected earlier. By three weeks they exhibit weight loss, piloerection, hunched posture and diarrhea. They also display defective hematopoiesis and immune function,

characterized by splenomegaly, lymphadenopathy, anemia and thymic atrophy. All *tcptp*$^{-/-}$ mice die at 3-5 weeks after birth [2].

In accordance with the higher level of TC-PTP expression in hematopoietic system, *tcptp*$^{-/-}$ mice have defects in both hematopoiesis and immune function. Three-week-old mice show a marked increase in spleen and lymph node cellularity. The red pulp of the *tcptp*$^{-/-}$ spleen is expanded, consistent with sequestration of red blood cells and correlating with the decreased hematocrit. The bone marrow cellularity decreases strikingly between days 17 and 21, accompanied by depletion of B220^{+} cells. Therefore, it seems that there is a switch toward extramedullary hematopoiesis, which cannot fully populate the various compartments. In terms of T cell numbers, there is a striking decrease in thymic double positive cells on day 21. However, the decrease in the absolute numbers of peripheral T cells is modest and the CD4+/CD8+ ratio is essentially unchanged [2].

In addition to lymphopoietic abnormalities, there are functional defects to the T and B cell compartments. The mitogen response of *tcptp*$^{-/-}$ splenocytes to ConA and LPS is severely impaired. There is also a defect in T cell dependent B cell responses as shown by decreased number of plaque-forming colonies from *tcptp*$^{-/-}$ spleens after immunization with sheep RBCs [2]. Part of the defect in mitogen assays may stem from the inherent proliferative defect of *tcptp*$^{-/-}$ cells [3]. Splenic T cells from *tcptp*$^{-/-}$ mice also produce less IL-2 than their *tcptp*$^{+/+}$ counterparts [253]; however, the addition of exogenous IL-2 does not rescue the phenotype. Stimulation with anti-CD3 is followed by normal tyrosine phosphorylation patterns and calcium flux, providing evidence that the immediate events downstream of TCR are not affected. Nevertheless, stimulating purified populations of *tcptp*$^{-/-}$ T or B cells partially rescues the phenotype, suggesting that although TC-PTP is necessary for rapid progression through cell cycle, there may be other factors contributing to the lack of proliferation in mixed cultures [254].

Recent data from our laboratory provide an explanation for the above findings. Culturing purified populations of Gr1^{+} *tcptp*$^{-/-}$ cells with *tcptp*$^{+/+}$ T cells produces a similar defect in proliferation in response to anti-CD3 as what is seen in *tcptp*$^{-/-}$ spleen cultures [254]. This is shown to correlate with NO production and is preventable by adding iNOS inhibitors or anti-IFN-γ to the culture medium. It is also contact-dependent, suggesting that other signals in addition to IFN-γ are necessary to induce NO production

by the $Gr1^+$ cells. One example would be the engagement of co-stimulatory molecules, as CD80 is upregulated on *tcptp*$^{-/-}$ $Gr1^+$ cells [254]. It must be noted that *tcptp*$^{+/+}$ $Gr1^+$ cells do not have the same effect on T cell proliferation *in vitro*, suggesting that the *tcptp*$^{-/-}$ cells have a higher activation status.

Bone marrow transplantation experiments suggested that the hematopoietic defect is not due to a problem with the stem cells but rather with the bone marrow stroma [2]. Irradiated normal recipients were fully reconstituted with *tcptp*$^{-/-}$ bone marrow, whereas *tcptp*$^{+/+}$ bone marrow graft did not rescue *tcptp*$^{-/-}$ recipients. Two months after the graft, there was no difference in T and B cell numbers in different organs between irradiated *tcptp*$^{+/+}$ mice that had received *tcptp*$^{+/+}$ or *tcptp*$^{-/-}$ bone marrow. Their hematocrit was normal, and there was no increase in the size of either spleen or lymph nodes. However, the functional defects seen in *tcptp*$^{-/-}$ mice were still present in the mice reconstituted with *tcptp*$^{-/-}$ bone marrow: mature T and B cells from the spleen of these animals failed to proliferate in response to mitogenic stimuli (ConA or anti-CD3 for T cells and LPS for B cells).

It is of note that there is no overt defect in *tcptp*$^{+/-}$ animals: they are generally healthy and fertile past 12 months of age. The percentages of various lymphoid and myeloid populations follow closely those seen in normal mice, and the mitogen and PFC responses are normal [2]. This indicates that one normal allele is sufficient to sustain hematopoiesis, proliferative T and B cell responses as well as T cell dependent B cell responses.

1.4.2.2 Protein tyrosine phosphatase 1B (ptp1b$^{-/-}$)

The PTP-1B deficient mice were created by two different laboratories with essentially similar phenotypes. The first report showed that when fed ad libitum, *ptp1b*$^{-/-}$ mice had lower blood glucose and lower blood insulin than the *ptp1b*$^{+/+}$ littermates [4]. They also displayed higher glucose tolerance and lower glucose levels after exogenous insulin. On high-fat diet, *ptp1b*$^{-/-}$ mice gained less weight and maintained better glucose tolerance and increased sensitivity to insulin when compared to *ptp1b*$^{+/+}$ mice. Their serum triglyceride levels were also lower. This reflected increased phosphorylation of the insulin receptor in

muscle and liver, suggesting that the loss of PTP-1B protected the mice from insulin resistance and therefore type II diabetes.

The second group reported that the absence of PTP-1B resulted most of all in a decrease in fat mass due to adipocyte hypotrophy [245]. The protection from diet-induced obesity stemmed from increased basal metabolic rate and increased glucose disposal, notably through decreased metabolic efficiency and dissipation of energy through heat. They further characterized the tissue specificity of the insulin response and showed that glucose uptake was enhanced in muscle but not in adipose tissue of *ptp1b*$^{-/-}$ mice. Together, the two reports provided a physiological confirmation to previous *in vitro* data implicating PTP-1B in insulin signalling. These findings were further confirmed by muscle-specific overexpression of PTP-1B, which was shown to cause insulin resistance [255]. Furthermore, a liver-specific rescue of PTP-1B greatly diminished insulin sensitivity of *ptp1b*$^{-/-}$ mice [256].

Klaman et al. noticed that circulating leptin levels were decreased in *ptp1b*$^{-/-}$ mice compared to *ptp1b*$^{+/+}$ mice, reflecting their decreased body fat mass [245]. This finding seemed to be in conflict with the increased energy expenditure and decreased body mass. Moreover, *ptp1b*$^{-/-}$ mice did not exhibit significantly increased food intake. Accordingly with the role of leptin on feeding and body mass control, the loss of PTP-1B was found to enhance leptin signalling [5, 125]. Injection of gold thioglucose (GTG) kills hypothalamic leptin-responsive neurones and renders mice hyperphagic and obese. Although the GTG-treated *ptp1b*$^{-/-}$ mice increased their food intake at levels comparable to the GTG-treated *ptp1b*$^{+/+}$ mice [125], their weight gain was only about half and their insulin sensitivity and circulating insulin levels remained normal. This suggested that although Stat3 phosphorylation was enhanced in the hypothalami of *ptp1b*$^{-/-}$ mice and PTP-1B was shown to dephosphorylate Jak2 downstream of the leptin receptor, regulating sensitivity to leptin was an important part but not the only means by which PTP-1B controlled body mass and obesity.

In another study, leptin deficient (*ob/ob)* mice were bred with *ptp1b*$^{-/-}$ mice with similar results [5]. Mice deficient for PTP-1B gained less weight on the *ob/ob* background, and their fat pads remained smaller. This was not due to differences in food intake, and correlated with better glucose tolerance and lower fasting glucose.

Interestingly, *ptp1b*$^{-/-}$ mice exhibited greater weight loss and restricted their food intake in response to exogenous leptin, whether on a lean or *ob/ob* background. This was shown *in vitro* to correlate with increased Stat3 phosphorylation downstream of leptin receptor, and association of Jak2 with PTP-1B in phosphotyrosine-specific fashion. In sum, these two studies showed that PTP-1B was able to control weight gain in leptin-dependent and –independent manner, and that even in obese mice, the loss of PTP-1B conferred protection against type II diabetes.

Based on the *ptp1b*$^{-/-}$ phenotype, PTP-1B has been proposed as a therapeutic target for type II diabetes and obesity (reviewed in [257, 258]). In contrast to transgenic mice, people who would require such treatment would already be obese and very likely diabetic. Furthermore, neither small inhibitors nor inhibitory RNA treatment would result in 100% ablation of PTP-1B activity. The studies by Cheng et al. and Zabolotny et al. [5, 125] indicated that *ptp1b*$^{+/-}$ mice were also somewhat protected as they exhibited an intermediate phenotype. Furthermore, the use of PTP-1B inhibitory RNA in *ob/ob* mice has yielded promising results, including decreased adiposity correlating with downregulation of adipogenic genes [259], as well as increased insulin sensitivity in liver and muscle [260]. Therapeutic use of antisense RNA is quite expensive, however, and the main drawback with small molecule inhibitors is their poor selectivity against TC-PTP, the closest relative to PTP-1B.

Genetic studies in humans have shown a correlation between certain non-coding mutations in *ptpn1* and type II diabetes in Caucasian and Hispanic populations [261, 262]. However, a recent study failed to detect any significant association between known haplotypes of *ptpn1* and diabetes [263]. The authors were unable to identify any significant differences between their study and that of Bento et al. [262] and concluded that the discrepancies were most likely due to unaccounted for modifier genes and possibly to the slight differences in subjects' body mass index (the BMI values in the most obese group in the North American study [262] were significantly higher than those in the Canadian-Scandinavian study [263]).

In sum, PTP-1B is best known as a metabolic regulator gene. Despite the role it is shown to have on Jak2-mediated signalling, no immune phenotype has yet been reported in *ptp1b*$^{-/-}$ mice.

1.5 TC-PTP AND INFLAMMATION

1.5.1 Systemic inflammation and septic shock

Inflammation, or the combination of redness, heat, pain and swelling traditionally used to describe an inflamed site (reviewed in [264]), is the basic response to injury and infection with the ultimate goal of eradicating the infection and repairing the injury. This requires multiple mechanisms to simultaneously detect infectious organisms and the extent of tissue injury, to promptly respond to the trauma, but also to shut down the response once the danger is past. When negative regulation is lacking or when the insult persists, the initially beneficial response may cause more damage than the infection or tissue trauma themselves.

The signal starts with resident sentinel cells, including tissue macrophages, dendritic cells and mast cells. These sentinel cells will then secrete cytokines and chemokines that will recruit in the first instance, neutrophils, followed by lymphocytes and monocytes. Activation of lymphocytes is dependent on antigen plus costimulatory signals from the resident antigen-presenting cells (APCs), whereas that of APCs requires cytokines plus either microbial products or, in certain cases, self-proteins released during tissue injury (see Toll-like receptors, section 1.2.2.1 and table 1.1).

Whereas neutrophils are key players in acute inflammation, monocyte/macrophages are both initiators of the inflammatory response and some of the major effectors of chronic inflammation. Their most potent activation signal is a combination of IFN-γ and LPS [265, 266], which will induce, for example, the production of tumor necrosis factor (TNF)-α [267] and nitric oxide [268]. TNF-α can attract lymphocytes [269] and neutrophils [270] as well as play an important role in tissue damage and sepsis [271-273]. Mice transgenic for TNF-α develop arthritis, whereas mice in which post-transcriptional regulation of TNF-α has been abolished through the deletion of the AU-rich element in the 3' untranslated region develop both arthritis and inflammatory bowel disease [274]. Moreover, antibodies against TNF-α have been approved for treatment in arthritis and Crohn's disease [275, 276], two conditions associated with chronic inflammation.

Nitric oxide is associated with tissue injury [277, 278] and immunosuppression [279-281]. When produced in combination with superoxide, it can form peroxynitrite

[282], which is a potent oxidizing and nitrating agent able to react with tyrosine to form 3-nitrotyrosine and thus interfere with regular protein function. NO production is also part of the negative feedback regulation of macrophage activation, as it has been shown to result in macrophage apoptosis [283, 284].

Mice with spontaneous inflammation have provided several clues to the understanding of the different components of inflammatory response and inflammatory disease. Although data obtained from mice cannot always be extrapolated into humans, the phenotypes of certain genetically modified mice correlate quite well with human mutations. A selection of transgenic, hypomorphic and knockout mice presenting chronic and/or acute inflammatory disease can be found in table 1.6.

Sepsis and septic shock result from a dysregulated inflammatory response [285], and involve bacterial toxins either in the form of LPS (for gram-negative bacteria) or superantigens and exotoxins (for gram-positive bacteria). Required host components include IFN-γ, TNF-α, IL-1 and (at least in gram-negative sepsis) TLR4. However, from a therapeutic point of view, blocking any of these cytokines may come too late [285]. Death from sepsis is due to multi-organ failure, mostly as a result of hypoperfusion and hypoxia.

1.5.2 Evidence for TC-PTP as a regulator of inflammatory responses

tcptp$^{-/-}$ mice display several hematopoietic defects, including proliferative defects and anaemia [2]. They die within 3-5 weeks from unknown causes: however, by three weeks of age they develop symptoms of wasting disease and septic shock, such as runting, hunched posture, piloerection and diarrhoea. They are immunosuppressed in that their lymphocytes do not proliferate in response to mitogenic stimuli, and they display thymic atrophy and a decrease in $CD4^+CD8^+$ double positive thymocytes. Similar signs characterize, for example, murine graft-versus-host disease (GVHD), a chronic systemic inflammatory syndrome [286].

Data from our laboratory and others have indicated that TC-PTP regulates cytokine signalling through Jak1, Jak3 [131] and Stat1 [128], Stat3 [129] and Stat5 [130]. One of the key cytokines involved in inflammatory responses is IFN-γ, and TC-PTP has been shown to downregulate IFN-γ-mediated signals [128, 131]. Moreover, GVHD is

Gene modified	Phenotype	Organs affected	Human?	Refs.
Fas (CD95) (*lpr*)	Lymphoproliferative disease; glomerulonephritis, vasculitis, synovitis, interstitial pneumonitis, dermatitis	Kidney, mesentery, joints, lungs, skin, vasculature	Yes [287]	[288, 289]
C1q	Glomerulonephritis	Kidney	Yes [290]	[291]
TGF-β	Macrophage, lymphocyte and neutrophil infiltration; gastric ulceration	Lung, heart, stomach, liver, spleen, lymph nodes, pancreas, colon, salivary glands, striated muscle	No?	[292, 293]
CTLA4	Lymphocyte, macrophage and granulocyte infiltration	Heart, pancreas, lung, bone marrow, liver, salivary glands, joints, blood vessels	Yes [294]	[295, 296]
SOCS1	Macrophage infiltration	Liver, lungs, pancreas, heart, skin	No?	[158, 159]
Cbl	Activated B- and T-cell infiltration	Salivary glands, pancreas, liver, intestine, lungs, kidney, heart, skeletal muscle, urinary bladder	No?	[297]
RelB	T-cell, granulocyte and macrophage infiltration; collagen deposition	Skin, lungs, liver, salivary glands, skeletal muscle, stomach, epididymis, ovaries, uterus	No?	[298]
SHP-1 (*me*)	Neutrophil abscesses, interstitial pneumonitis	Skin, lungs	No?	[194, 195, 299]

Table 1.6. – Mouse models of inflammation.

A selection of genetically modified mice presenting multi-organ inflammation. Adapted from [264].

associated with macrophage priming, which renders them more sensitive to exogenous or endogenous LPS [286]. Primed or activated macrophages are then able to upregulate TNF-α and iNOS, especially after further stimulation with LPS [286, 300]. IFN-γ is the major macrophage priming cytokine [265]; *tcptp*$^{-/-}$ mice also display increased numbers of splenic macrophages (F4-80^{+} cells) [2].

Therefore, we hypothesized that *tcptp*$^{-/-}$ macrophages might be primed *in vivo* due to the loss of negative regulation on IFN-γ signalling. They would produce increased amounts of TNF-α and NO, causing tissue pathology and contributing to the death of *tcptp*$^{-/-}$ animals. The amount of IFN-γ present would not necessarily need be augmented as, according to our hypothesis, even small amounts of IFN-γ would result in macrophage activation in the absence of TC-PTP. The results of these studies were published in Blood in 2004 [301] and presented in Chapter 2 of this thesis.

1.6 PTP-1B AND MYELOPOIESIS

1.6.1 Overview of monocyte/macrophage development

All myeloid cells, whether they be erythrocytes, platelets or macrophages, arise from a common myeloid progenitor cell (CMP), identified by Akashi et al. in 2000 [302]. The CMP will then differentiate into megakaryocyte/erythrocyte (MEP) or granulocyte/monocyte progenitors (GMP). These clonogenic progenitor cells will eventually give rise to mature myeloid cells, depending on the cytokine environment and transcription factors induced. A simplified view of myelopoiesis together with cell surface markers for different populations can be found in figure 1.6.

It is generally accepted that IL-3 and GM-CSF are multilineage cytokines that can stimulate both granulocytic and monocytic differentiation. Conversely, stimulation with G-CSF is known to result in granulocyte formation, whereas CSF-1 (or M-CSF) will favour monocyte formation (reviewed in [303]). GM-CSF deficient mice have normal numbers of myeloid cells [304], but are more sensitive to pulmonary infections. Mice with no G-CSF are neutropenic (20-30% normal levels), a defect which can be reversed by exogenous G-CSF, and more susceptible to infection with *Listeria monocytogenes* [305]. Mice lacking both G-CSF and GM-CSF are more severely neutropenic and show

increased perinatal lethality [306]. Mice deficient in CSF-1 (*op/op*) have greatly reduced numbers of macrophages and are osteopetrotic due to decreased osteoclast activity [307, 308]. Lack of both GM-CSF and CSF-1 results in frequent pulmonary infections accompanied by neutrophilia, but the macrophage phenotype is no more severe than in *op/op* mice [309]. These data show that although different myeloid lineages favour certain CSFs, *in vivo* CSFs display some redundant function.

Hematopoietic growth factors act through the induction of lineage specific gene expression, which is largely mediated by specific transcription factors. The two major myeloid transcription factors are PU.1 and C/EBPα [303, 310]. However, neither is myeloid specific, but can also be found in, e.g., B lymphocytes (PU.1) or non-hematopoietic tissues (C/EBPα). Both are expressed in myeloid precursors and are able to activate the transcription of CSF receptor genes.

Mice deficient in PU.1 lack both monocytes and granulocytes [311, 312], and *in vitro* studies have shown that PU.1 is necessary for the terminal differentiation of both lineages [313]. Interestingly enough, a recent report indicated that the duration of PU.1 expression may be crucial in determining myeloid fate: transient expression would favour granulocytic development, whereas long term expression would block granulocytic differentiation and result in monocyte formation [314]. This was corroborated by another study, which showed that the loss of one allele of PU.1 increased the proportion of granulocytes in G-CSF -deficient mice [315]. Therefore, PU.1 dosage appears to play an important role in granulocytic versus monocytic differentiation.

In the hematopoietic system, C/EBPα is predominantly expressed in immature granulocytes [310]. Accordingly, the loss of C/EBPα results in absence of mature neutrophils due to lack of G-CSFR expression [316]. These mice had high levels of circulation myeloblasts and normal monocytic and lymphoid development. G-CSF signalling also has the effect of increasing C/EBPα levels, a fact that inhibits monocytic and favours granulocytic differentiation [315]. C/EBPα is also often mutated in AML [317], which is characterized by immature myelocytes.

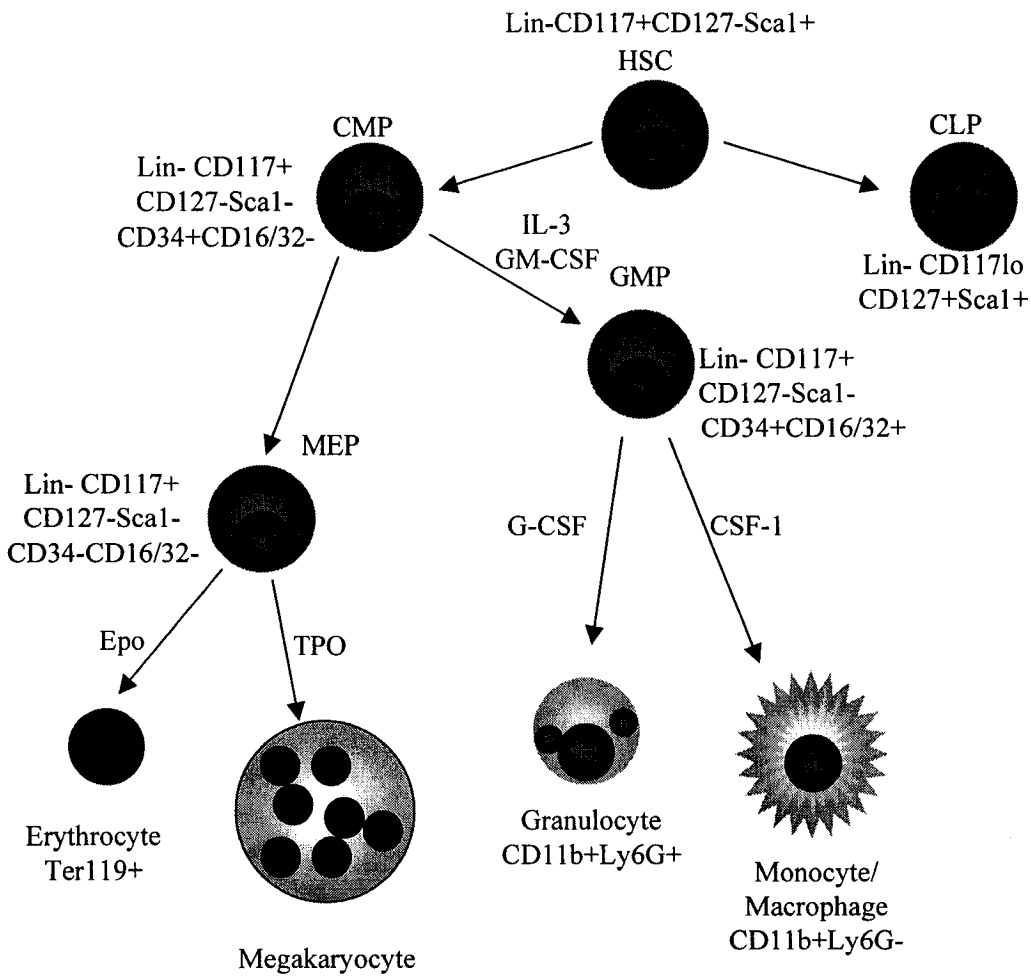


Figure 1.6 - Myeloid differentiation.

An overview of the development of various myeloid lineages, including the expression of surface markers and the role of major hematopoietic growth factors.

Other transcription factors that influence myeloid development include early growth response (Egr)-1 [318] and IFN regulatory factor (IRF)-8 [319]. Overexpression of Egr-1 favoured monocytic development at the expense of granulocytes [320], and more recently was shown to mediate terminal differentiation by antagonizing c-Myc [321]. IRF-8 interacts with PU.1 [322] and mice deficient in IRF-8 develop granulocytosis and ultimately a syndrome similar to chronic myelogenous leukemia [323].

1.6.2 Evidence for PTP-1B in myeloid development

Despite the fact PTP-1B has no known role in the immune system, it is expressed in hematopoietic cells [324, 325]. It has also been shown to dephosphorylate Jak2 and Tyk2 [7] as well as Stat5 [126]. Therefore, it would be reasonable to propose that PTP-1B would affect hematopoietic cytokine signalling, and consequently immune development and function.

Jak2 is activated downstream of a multitude of cytokine receptors, including G-CSF and GM-CSF (see table 1.2). Moreover, the receptor for CSF-1 belongs to the same RTK subfamily as PDGFR, a known substrate of PTP-1B [14]. PTP-1B therefore has the potential to regulate myelopoiesis at several different levels. It has also been published that the levels of PTP-1B are increased in cell lines derived from CML patients [252, 326]. The oncoprotein p210Bcr-Abl present in these cells is a substrate for PTP-1B; however, it is not known what the reason for the upregulation of PTP-1B expression is in CML, nor are the functional consequences of such expression clear. Based on this information, we wished to investigate the role of PTP-1B in myeloid development, and the results of these studies are currently in press in the Proceedings of the National Academy of Sciences and presented in Chapter 3.

Concurrently with the work on PTP-1B in myelopoiesis, we also discovered that it plays a role in B cell development. The absence of PTP-1B resulted in increased number of $B220^{+}$ cells in the bone marrow and lymph nodes, and increased the occurrence of B lymphomas in $p53^{-/-}$ mice. These data were published earlier this year in Cancer Research [327].

1.7 COMPLEMENTARY ROLE OF TC-PTP AND PTP-1B

1.7.1 Structural similarities and common substrates

TC-PTP and PTP-1B bear a high degree of structural similarity (72% amino acid sequence identity within the catalytic domain) [328], and functional identity [329]. Moreover, the majority of small molecule inhibitors synthesized against PTP-1B display no or very limited selectivity against TC-PTP [8, 257, 258]. Given that these inhibitors are currently under consideration for treatment of type II diabetes, it becomes important to know whether TC-PTP and PTP-1B function *in vivo* is redundant, and what the consequences would be of simultaneously interfering with the action of both. Therefore, we opted for the generation of *tcptp*$^{-/-}$*ptp1b*$^{-/-}$ double mutant mice.

TC-PTP and PTP-1B have four common published substrates, EGFR, PDGFR, IR and Stat5 (see table 1.7). In addition, together they dephosphorylate all members of the mammalian Jak family. Physiological evidence for either TC-PTP or PTP-1B as regulators of EGFR or PDGFR is slight. Although PTP-1B is well known for its ability to regulate IR signalling, and *ptp1b*$^{-/-}$ mice are more sensitive to insulin, we have not found any *in vivo* evidence that IR signalling would be affected by the loss of TC-PTP (M.L. Tremblay, personal communication). It has been shown that decreasing TC-PTP levels by RNA interference in *ptp1b*$^{-/-}$ fibroblasts enhanced insulin-mediated signalling along the protein kinase B but not MAPK pathway [233]. However, the authors did not report any physiological consequence of this prolonged signalling.

Given the data presented in Chapter 3, we felt that it would be more likely that PTP-1B and TC-PTP collaborate in cytokine signalling. Table 1.8 summarizes the various cytokine pathways that TC-PTP and PTP-1B could cooperatively regulate based on the known Jak/Stat substrates for both enzymes. The simultaneous loss of the two enzymes would presumably result in prolonged and increased activation of the pathway, similar to ectopic expression of the activating cytokine, which is why I have included the phenotypes of the mice transgenic for the various cytokines. Out of these candidate pathways, only interferon signalling has been published to be influenced by both TC-PTP and PTP-1B. Moreover, given that IFN-γ is a macrophage activating, inflammatory type cytokine, and that the results presented in Chapters 2 and 3 demonstrate that both TC-PTP and PTP-1B can modulate macrophage development and/or activation, we hypothesized

that IFN-γ-mediated macrophage activation might well be one common denominator of TC-PTP and PTP-1B function. The results of our studies are presented in Chapter 4.

Substrate	Evidence for TC-PTP	Evidence for PTP-1B	Refs.
EGFR	Substrate-trapping	Substrate-trapping; hyperphosphorylation in -/- MEFs	[227, 240-242]
PDGFR	Hyperphosphorylation in -/- embryos; *in vitro* dephosphorylation	Substrate-trapping; hyperphosphorylation in -/- MEFs	[232, 241-243]
IR	Substrate-trapping; hyperphosphorylation in -/- MEFs	Substrate-trapping; hyperphosphorylation in -/- tissues; increased insulin sensitivity in -/- mice	[4, 233, 234, 244, 245]
Stat5	Decreased phosphorylation after PTP overexpression; *in vitro* dephosphorylation	Decreased phosphorylation after PTP overexpression; *in vitro* dephosphorylation	[126, 130]
Jak family	Substrate-trapping; hyperphosphorylation in -/- cells	Substrate-trapping; hyperphosphorylation in -/- cells and tissues; increased leptin and GH sensitivity in -/- mice	[5-7, 125, 131]

Table 1.7 – Common substrates for TC-PTP and PTP-1B.

A list of substrates reported to be dephosphorylated by both TC-PTP and PTP-1B, including the methods for substrate identification. Substrate-trapping refers to phosphotyrosine-specific association with catalytically inactive mutant [117]. MEFs, murine embryonic fibroblasts; GH, growth hormone.

Cytokine	Jaks and Stats	Transgenic phenotype	Refs.
IFN-γ	Jak1, Jak2, Stat1	Tissue-specific inflammation/ autoimmune disease	[330-333]
IFN-α/β	Jak1, Tyk2, Stat1, Stat2	Tissue-specific inflammation/ autoimmune disease	[334, 335]
IL-6/LIF/OSM/CNTF	Jak1, Jak2, Stat3	Tissue-specific inflammation/ autoimmune disease	[336-339]
IL-10	Jak1, Tyk2, Stat3	Autoimmune diabetes (pancreas); suppression of inflammation/Th1 responses (T cells)	[340-343]

Table 1.8 – Cytokine pathways potentially regulated by both TC-PTP and PTP-1B.

A summary of the cytokines that utilize Jak1 and either Jak2 or Tyk2, including phenotypes of tissue-specific transgenic animals.

2 CHAPTER II – T CELL PROTEIN TYROSINE PHOSPHATASE DELETION RESULTS IN PROGRESSIVE SYSTEMIC INFLAMMATORY DISEASE

2.1 ABSTRACT

The deregulation of the immune response is a critical component in inflammatory disease. Recent *in vitro* data show that T Cell Protein Tyrosine Phosphatase (TC-PTP) is a negative regulator of cytokine signaling. Furthermore, *tc-ptp*$^{-/-}$ mice display immune defects and die within five weeks of birth. We report here that *tc-ptp*$^{-/-}$ mice develop progressive systemic inflammatory disease as shown by chronic myocarditis, gastritis, nephritis and sialadenitis as well as elevated serum interferon-γ. The widespread mononuclear cellular infiltrates correlate with exaggerated interferon-γ, tumor necrosis factor-α, interleukin-12 and nitric oxide production *in vivo*. Macrophages grown from *tc-ptp*$^{-/-}$ mice are inherently hypersensitive to lipopolysaccharide, which can also be detected *in vivo* as an increased susceptibility to endotoxic shock. These results identify T Cell Protein Tyrosine Phosphatase as a key modulator of inflammatory signals and macrophage function.

2.2 INTRODUCTION

Protein phosphorylation, the most common post-translational modification in mammalian cells, is used as a rapid means of modulating enzyme function. The control of cellular phosphorylation levels depends on the reciprocal activity of kinases and phosphatases, and defects in either component can have widespread effects on cell function. T Cell Protein Tyrosine Phosphatase (TC-PTP) is a nuclear phosphatase that is strongly expressed in the hematopoietic system. We have previously shown that it is essential for coordinate immune function and development [2]. More recently, it has been identified as a negative regulator of signal transduction associated with cytokine signaling through Janus kinases (Jak) –1 and –3 [131] as well as several members of the signal transducers and activators of transcription (Stat) family [128-130].

The *tc-ptp*$^{-/-}$ mice show normal genotypic frequency at birth and appear to be physically normal until 10-14 days of age, after which their growth is retarded [2, 253]. Although the total number of peripheral T and B cells remains relatively unchanged, the *tc-ptp*$^{-/-}$ mice are severely immunosuppressed. They are unable to mount T cell dependent B cell responses [2], and their splenocyte response to mitogens is totally suppressed. Moreover, thymic double positive T cells decline sharply with age and stromal defects in the bone marrow result in depletion of B cell precursors by three weeks of age [2]. The *tc-ptp*$^{-/-}$ mice progressively develop symptoms of wasting disease [271-273], including runting, hunched posture, diarrhea and weight loss [2], and die at 3-5 weeks of age.

Severe immune suppression accompanied by wasting disease is a hallmark of graft versus host disease (GVHD), which is characterized by a loss of T cell proliferative responses and the systemic priming of macrophages [286, 300, 344, 345]. Unstimulated macrophages from normal animals respond to an interferon-γ (IFN-γ) plus lipopolysaccharide (LPS) activation by producing tumor necrosis factor-α (TNF-α) [265, 267] and nitric oxide (NO) [266, 268]. Both are mediators of tissue damage [271-273, 277, 346] that play an important role in septic shock. Excess NO has also been shown to cause immune suppression [281, 347, 348]. The immune suppression and apparent onset of inflammatory symptoms together with the reported increase in number of splenic macrophages [2] in *tc-ptp*$^{-/-}$ mice and the *in vitro* data for a role in cytokine signalling

[128, 131] led us to hypothesize that *in vivo* deletion of TC-PTP may result in overproduction of cytokines and development of inflammatory disease, which would account, at least in part, for the early mortality seen in *tc-ptp*$^{-/-}$ mice.

In this report we show that the expression of IFN-γ, TNF-α and inducible nitric oxide synthase (iNOS) is upregulated *in vivo* in *tc-ptp*$^{-/-}$ mice. A significant increase can be detected as early as three days after birth, prior to the onset of any overt disease. The overproduction of cytokines contributes to the priming of inflammatory cells *in vivo*, as shown by an increase in sensitivity to LPS both *in vitro* and *in vivo*. Furthermore, *tc-ptp*$^{-/-}$ macrophages are inherently hypersensitive to LPS. We also demonstrate the presence of a widespread mononuclear infiltrate in several non-lymphoid organs. Taken together, our observations indicate that TC-PTP is a key negative regulator of cytokine signaling and inflammatory disease.

2.3 MATERIALS AND METHODS

Mice. *Tc-ptp*$^{-/-}$ mice were obtained from heterozygous matings and genotyped as previously described [2]. *Tc-ptp*$^{+/+}$ and *tc-ptp*$^{+/-}$ littermates were used as controls. All mice were kept in specific pathogen free housing in the animal care facility. Protocols were approved by the McGill animal care ethics committee.

Reagents, antibodies, and media. Cell culture reagents were purchased from Invitrogen (Burlington, ON) unless otherwise specified. Lipopolysaccharide was obtained from Calbiochem (San Diego, CA), and prepared as previously described [286]. Recombinant murine interferon-γ was purchased from Invitrogen and ConcavalinA from Pharmacia (Baie-d'Urfé, QC). TC-PTP was detected with the monoclonal mouse antibody clone 4F4. The polyclonal rabbit anti-iNOS antibody was purchased from Upstate Biotechnology (Lake Placid, NY) and polyclonal rabbit antiserum for calnexin was kindly provided by Dr. J.J. Bergeron (McGill University, Montreal, QC). Secondary antibodies used were horseradish peroxidase-conjugated anti-mouse (Jackson Immunoresearch) and anti-rabbit immunoglobulin (Upstate Biotechnology, Lake Placid, NY) as appropriate. FITC-conjugated anti-Gr-1, PE-conjugated anti-CD14 and APC-conjugated anti-Mac1 antibodies for flow cytometry were purchased from BD Biosciences Pharmingen (Mississauga, ON).

Quantification of mRNA. Total RNA was extracted from tissues or cultured cells using TRIzol (Invitrogen, Burlington, ON) according to the manufacturer's specifications. mRNA was quantified using the QuantiTect™ SYBR® Green RT-PCR kit (Qiagen, Mississauga, ON) and the Light Cycler® Real-Time PCR machine (Roche Diagnostics, Laval, QC). The samples were first incubated at 50°C for 20 min to synthesize first strand cDNA, after which they were heated to 95°C for 15 min in order to activate the Taq polymerase. Conditions for amplification are described below. After each cycle total fluorescence was determined as a measure of double-stranded DNA in the sample, and the data were used as the basis for quantification. The identity of the PCR products was confirmed by melting curve analysis. Data were analyzed using the LCDA software (Roche Diagnostics, Laval, QC). GAPDH was used as a housekeeping message, and the message of interest to GAPDH ratio was taken as the relative expression level. To compare between organs and between age groups, the *tc-ptp*$^{+/+}$ expression level was always set as one, and the *tc-ptp*$^{-/-}$ level was expressed as the fold increase or decrease as compared to age-matched *tc-ptp*$^{+/+}$ mice.

Primers. The following oligonucleotides were synthesized at the Sheldon Biotechnology Centre (McGill University, Montreal, Quebec) and used as follows: GAPDH FWD 5'-AATGGTGAAGGTCGGTGTGAAC-3' and GAPDH REV 5'-TGGAAGATGGTGAT GGGCTTC-3' (15s 95°C, 10s 57°C, 20s 72°C; 30 cycles); TNFα FWD 5'-CCTGTAGCC CACGTCGTAGC-3' and TNFα REV 5'-TTGACCTCAGCGCTGAGTTG-3' (15s 95°C, 8s 57°C, 25s 72°C; 35 cycles); iNOS FWD 5'-GCCGCATGAGCTTGGTGTTTG-3' and iNOS REV 5'-TGATAACGTTTCTGGCTCTTGAG-3' (15s 95°C, 9s 62°C, 25s 72°C; 40 cycles); IFNγ FWD 5'-TGGAGGAACTGGCAAAAGGATG-3' and IFNγ REV 5'-CGCTTCCTGAGGCTGGATTC-3' (15s 95°C, 10s 61°C, 25s 72°C; 30 cycles); IL-12 (p40) FWD 5'-AGATGACATCACCTGGACCTCAG-3' and IL-12 (p40) REV 5'-ACGTGAACCGTCCGGAGTAA-3' (15s 95°C, 8s 62°C, 30s 72°C; 35 cycles).

Histology. Following euthanasia, complete autopsies were performed on all animals. All of the organs from each animal were place in an individual bottle of 10% buffered formalin for fixation. The bottles were labeled in code, such that the reviewing pathologist had no knowledge of the animal's genotype or age. After fixation, the tissue from each bottle was placed in a single cassette and processed. After overnight

dehydration and impregnation of the tissue by paraffin, all of the tissue from each animal was embedded in a paraffin block and serially sectioned. The 5 micron thick sections were stained with hematoxylin and eosin (H and E) and examined by light microscopy. A minimum of four H and E sections was reviewed from each animal; in several animals, serial sections (> fifteen) were cut, stained and examined.

Measurement of serum IFN-γ. 19 to 21-day-old mice were injected i.p. with 150 µl sterile PBS containing 0 µg, 4 µg or 8 µg LPS. Blood samples were collected 5 hours post-injection, allowed to clot and separated. Serum IFN-γ was measured with OptEIA™ mouse IFN-γ ELISA (BD Pharmingen). In brief, 96-well plates were coated with a capturing monoclonal anti-mIFN-γ antibody overnight. The amount of bound cytokine from serum samples was measured using a second anti-mIFN-γ antibody with a different specificity and horseradish peroxidase/tetramethylbenzidine (TMB) –based detection system (BD Pharmingen). Absorbance was measured at 450nm on an ELISA plate reader (SLT Labinstruments, Salzburg, Austria) and compared to a recombinant mIFNγ standard curve.

Spleen cultures. Splenocytes were harvested from 14, 19 and 21-day-old mice, passed through stainless steel mesh, and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 10 U/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO_2. For iNOS mRNA, cells were stimulated with 100 U/ml IFNγ and 10 ng/ml LPS for 4 hours.

Splenic T cells were purified by positive selection for CD4 and CD8 using the EasySep™ magnetic nanoparticles (StemCell Technologies, Vancouver, BC). The PE-conjugated antibodies were obtained from BD Biosciences. For IFN-γ mRNA, total spleen cells or CD4/CD8 -selected T cells were cultured in medium supplemented with 2.5 µg/ml ConA for 16 hours.

Spleen-derived macrophages were obtained essentially as previously described [349]. In brief, spleen cells were cultured for seven days in 12-well plates at $1.6x10^6$ cells / well and in medium supplemented with 30% L-929 conditioned medium and 10% FBS. The supplemented medium was refreshed on day four. The L-929 medium was replaced with unsupplemented medium for 24 hrs before stimulation with IFN-γ and/or LPS.

Nitrite assay. Measurement of NO production was performed essentially as described previously [300]. In brief, spleen-derived macrophages were cultured in RPMI 1640 supplemented with 10% FBS, 10 U/ml penicillin and 100 μg/ml streptomycin and additional activators, where indicated. To measure the concentration of nitrite in the supernatant after 48 hours, 100 μl Griess reagent was added to 100 μl supernatant, and the optical density was read at 550nm on an ELISA plate reader (SLT Labinstruments). The absorbance values were then compared to a standard curve of known concentrations of sodium nitrite.

2.4 RESULTS

2.4.1 Upregulation of inflammatory mediators in tc-ptp$^{-/-}$ mice.

Recent reports showed that signaling components downstream of the IFN-γ receptor were hyperphosphorylated in *tc-ptp*$^{-/-}$ cells *in vitro* [128, 131]. Since IFN-γ is an important regulator of inflammatory responses, we examined the effect that the lack of TC-PTP would have on the expression of IFN-γ, as well as two IFN-γ responsive genes, TNF-α and iNOS, *in vivo*.

First we wished to study *tc-ptp* $^{-/-}$ mice at late stage of disease. We extracted RNA from different tissues of 20-day-old mice, and analyzed it by real time quantitative RT-PCR to determine the transcript levels *in vivo*. There was a significant increase in the level of TNF-α mRNA in the liver (approx. 15-fold) as well as in the salivary gland (27-fold) (Fig. 2.1*a*), mirrored by a respective four to ten-fold upregulation of iNOS mRNA (Fig. 2.1*b*). We observed similar results for IFN-γ in the liver and a particularly large increase in the salivary gland (120-fold) (Fig. 2.1*c*).

The increased expression of iNOS was not restricted to mRNA but was also reflected at the protein level (Fig. 2.2). A 125kDa band was clearly evident in *tc-ptp*$^{-/-}$ liver and salivary gland, while there was no detectable iNOS present in the corresponding *tc-ptp*$^{+/+}$ tissues. The enzyme was present in the thymus of both *tc-ptp*$^{+/+}$ and *tc-ptp*$^{-/-}$ animals [344]. Membranes were stripped and reprobed for TC-PTP to confirm genotype and for calnexin, a resident protein within the endoplasmic reticulum, to ensure equal loading of protein in each lane.

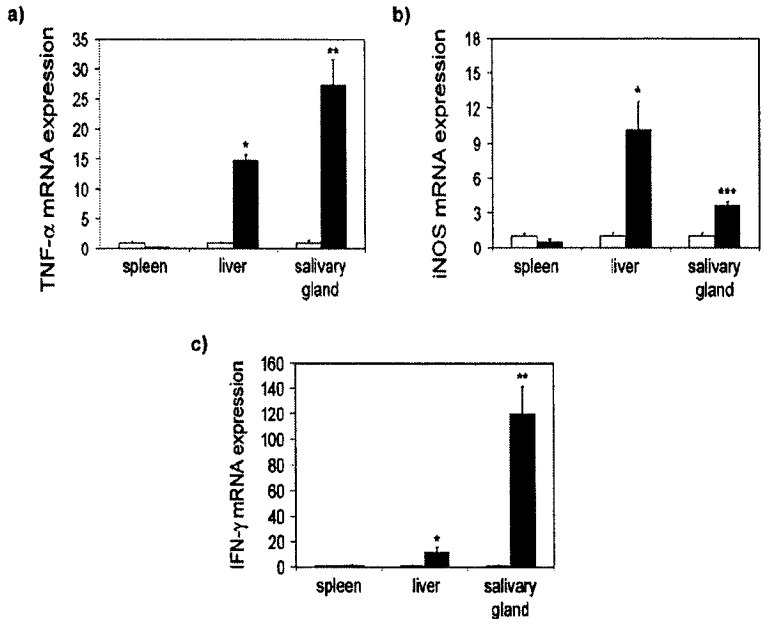


Figure 2.1 - Increased transcription of inflammatory mRNA on day 20 in tcptp-/- animals.

mRNA levels for *A)* TNF-α, *B)* iNOS and *C)* IFN-γ in tcptp-/- mice (■) and their wild-type littermates (□) at 20 days after birth. Bars represent the mean ± SE from four to five mice with tcptp-/- values shown as fold increase from those of age-matched tcptp-/- mice. Statistical significance was determined by a two-tailed unpaired Student t test. *P<0.05; **P<0.005; ***P<0.001 tcptp-/- compared to tcptp+/+.

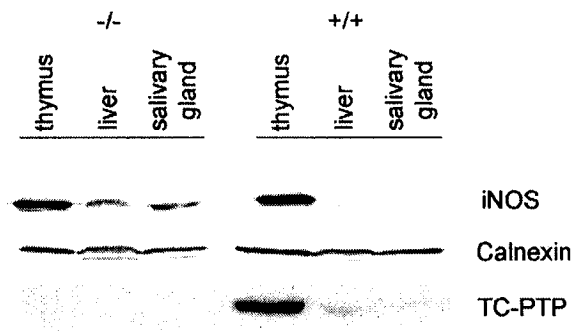


Figure 2.2 - Upregulation of iNOS protein in tcptp-/- mice.

Western blots showing TC-PTP and iNOS expression in the thymus, liver and salivary gland of three-week-old mice. Calnexin was used as a loading control. Data shown are representative of three separate experiments.

At birth, *tc-ptp*$^{-/-}$ mice appear normal and do not develop symptoms until 10-14 days of age[2]. It is possible that the increased activation of inflammatory mediators is secondary to another defect brought on by the lack of TC-PTP and not a direct effect of its deletion. To further explore this possibility, we determined the expression levels of IFN-γ, TNF-α and iNOS in thymus, liver and salivary gland at the perinatal stage (three days after birth) and at 12-14 days after birth which coincides with the time that overt symptoms first appear. An upregulation in expression of TNF-α and iNOS was already apparent in the liver at three days after birth (Fig. 2.3*a*, lower panel). There was a corresponding increase in IFN-γ expression as well. We did not detect any appreciable difference within the thymus (Fig. 2.3*a*, upper panel), and the expression levels in the spleen were not significantly increased (data not shown). The data from the salivary gland showed considerable variation from mouse to mouse (data not shown).

At two weeks of age, a significant increase in the TNF-α and iNOS transcripts was detected in the *tc-ptp*$^{-/-}$ thymus (Fig. 2.3*b*, upper panel), accompanied by an even greater level of upregulation in the liver (Fig. 2.3*b*, middle panel). Furthermore, TNF-α was upregulated in the salivary gland of 14-day-old *tc-ptp*$^{-/-}$ pups (Fig. 2.3*b*, lower panel). The increase in IFN-γ mRNA in the liver was already over 20-fold and the signal in the salivary gland had reached similar levels to those seen on day 20 (over 100-fold increase). The inflammatory reaction appears to be inherent, since it can be detected as early as three days after birth, and quickly becomes amplified with time.

2.4.2 Increased IFN-γ production is preceded by an upregulation of IL-12.

IL-12 is an IFN-γ-inducing cytokine that is normally produced by macrophages in response to microbial components [350]. When we measured the levels of the inducible subunit p40 in three-day-old pups, we could see a massive upregulation in the *tc-ptp*$^{-/-}$ liver as well as to a lesser extent in the spleen (Fig. 2.4*a*). At two weeks, there was a comparable increase in the *tc-ptp*$^{-/-}$ spleen, liver and salivary gland (Fig. 2.4*b*). Interestingly, the level of p40 mRNA returned to normal levels in all organs by day 21

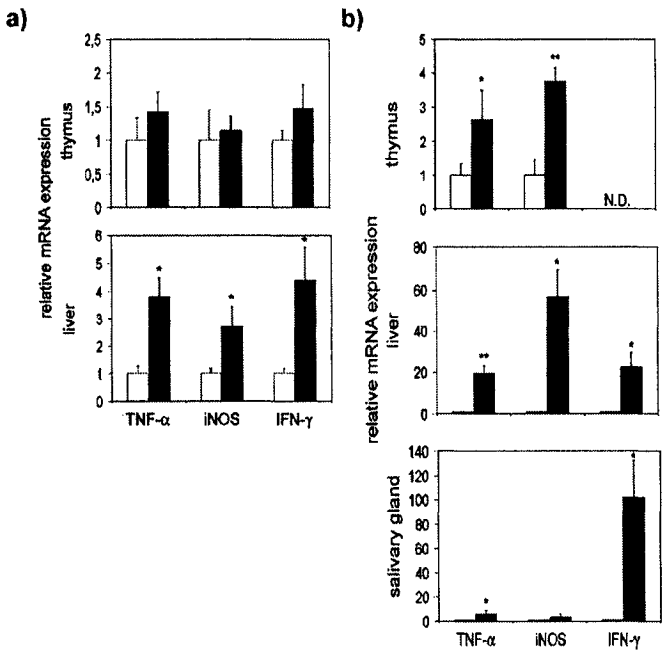


Figure 2.3 - Upregulation of TNF-α and iNOS mRNA precedes the symptoms of inflammatory disease and can be detected as early as day 3.

mRNA levels for TNF-α, iNOS and IFN-γ in the thymus, liver and salivary gland at ***A)*** three days and ***B)*** two weeks after birth. Bars represent the mean values ± SE from four to five mice, with tcptp-/- values (■) shown as fold increase from those of age-matched tcptp+/+ mice (□). Statistical significance was determined by a two-tailed unpaired Student t test. * $P < 0.05$; ** $P < 0.005$ tcptp-/- compared to tcptp+/+.

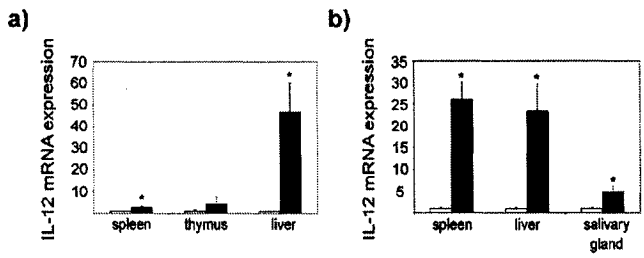


Figure 2.4 - Increased IL-12 p40 expression in tcptp-/- mice.

mRNA levels for IL-12 p40 subunit in ***A)*** spleen, thymus and liver at three days and ***B)*** spleen, liver and salivary gland at two weeks after birth. Bars represent the mean values ± SE from four mice, with tcptp-/- values (■) shown as fold increase from those of age-matched tcptp+/+ mice (□). Statistical significance was determined by a two-tailed unpaired Student t test. * $P < 0.05$ tcptp-/- compared to tcptp+/+.

(data not shown), suggesting that some negative feedback mechanism may be still in place despite the overall dysregulation of inflammatory cytokines.

We reported previously that *tc-ptp*$^{-/-}$ splenic T cells display a severe proliferation defect in response to mitogenic stimulation [2, 254]. The strong IFN-γ production observed in *tc-ptp*$^{-/-}$ mice suggested that the T cell defect may be restricted to proliferation and would not affect cytokine production. We purified T cells from the spleen using antibodies against CD4 and CD8 and stimulated them overnight with the T cell mitogen ConcavalinA. There was no difference in IFN-γ production between unstimulated *tc-ptp*$^{+/+}$ and *tc-ptp*$^{-/-}$ cells, but ConcavalinA-activated *tc-ptp*$^{-/-}$ T cells produced over five times more IFN-γ than their *tc-ptp*$^{+/+}$ counterparts on a per cell basis (Fig. 2.5). Similar results were obtained using whole spleen cultures. This indicates that proliferation and cytokine production are two independent events and that TC-PTP has opposite effects on the two.

2.4.3 Development of systemic inflammation in tc-ptp$^{-/-}$ mice.

The presence of inflammatory cytokines in the salivary glands of *tc-ptp*$^{-/-}$ mice led us to examine the presence of an inflammatory infiltrate in non-lymphoid organs. A total of nine *tc-ptp*$^{-/-}$ mice as well as 20 *tc-ptp*$^{+/+}$ and *tc-ptp*$^{+/-}$ littermates were euthanized at one, two, or three weeks after birth and tissue from each mouse was evaluated for pathology. At one week, subtle, focal infiltration of the salivary gland as well as small lymphocytic foci in the stomach could be detected in some *tc-ptp*$^{-/-}$ mice. In addition to the infiltration of the stomach and salivary gland, chronic lymphocytic myocarditis was observed in two-week-old mice. All three 21-day-old *tc-ptp*$^{-/-}$ mice featured combined chronic myocarditis (Fig. 2.6*a*), chronic gastritis (Fig. 2.6*b*) and chronic sialadenitis (Fig. 2.6*c*); in two of these, lymphocytic infiltrates were also present in the kidney (Fig. 2.6*d*); in one, chronic panniculitis was found in a random section of skin. The heart sections showed evidence of patchy myocyte degeneration and necrosis, consistent with chronic lymphocytic myocarditis, and it is not inconceivable that some *tc-ptp*$^{-/-}$ mice would die from a cardiac arrhythmia. Chronic gastritis was accompanied with glandular injury in at least two cases. One animal showed foci of lymphocytic infiltration of the renal cortex. Another had

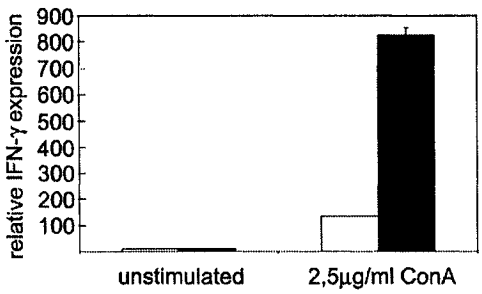


Figure 2.5 - Increased IFN-γ production upon mitogenic stimulation by tcptp-/- splenic T cells.

mRNA levels for IFN-γ from splenic T cells from 19-day-old mice. The cells were either left unstimulated or stimulated overnight with 2.5 µg/ml Concavalin A. Bars represent the mean values ± SE from triplicate cultures, with tcptp-/- values (■) shown as fold increase from those of age-matched tcptp+/+ cells (□). Three separate experiments were performed and all gave similar results. The results shown are from one representative experiment.

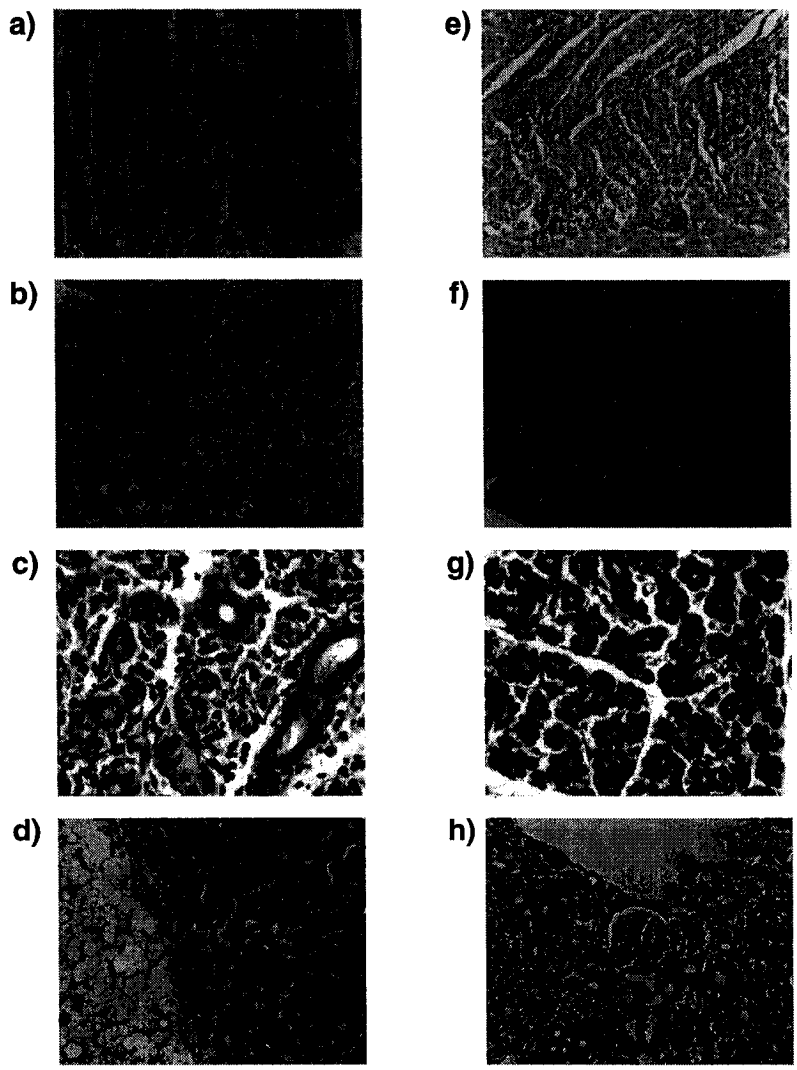


Figure 2.6 - Systemic inflammation in tcptp-/- mice.

A) Light micrograph of myocardium of tcptp-/- mouse age D21 featuring interstitial edema, prominent interstitial mononuclear cellular infiltrate and patchy myocyte degeneration and necrosis. (H and E section x200). ***B)*** Light micrograph of gastric mucosa of tcptp-/- mouse age D21 featuring dense mononuclear cellular infiltrate with patchy destruction of gastric epithelium. (H and E section x 200). ***C)*** Light micrograph of parotid gland of tcptp-/- mouse age D21 demonstrating mononuclear cellular infiltrate. (H and E section x200). ***D)*** Light micrograph of kidney and adjacent adipose tissue of tcptp-/- mouse age D21 demonstrating mononuclear cellular infiltrate in the superficial renal cortex and adjacent perinephric adipose tissue. (H and E section, x200). ***E), F), G)*** and ***H)*** Light micrographs of myocardium, gastric mucosa, parotid gland and kidney, respectively, of tc-ptp+/- mice age D21 showing normal histology.

lymphocytic infiltrates in the perirenal adipose tissue that extended into the subjacent renal cortex. The liver sections revealed a mild degree of extramedullary hematopoiesis in the younger animals (day 6-7). Sections from one liver demonstrated patchy hepatocellular necrosis. Overall the number and severity of inflammatory lesions increased with age, consistent with a progressive disease. The results are summarized in Table 2.1.

Neither *tc-ptp*$^{+/+}$ nor *tc-ptp*$^{+/-}$ littermate controls demonstrated any histological evidence of chronic lymphocytic myocarditis (Fig. 2.6*e*) or gastritis (Fig. 2.6*f*), and no lymphocytic infiltration was observed in the salivary gland (Fig. 2.6*g*), renal parenchyma (Fig. 2.6*h*), perirenal adipose tissue or skin. The liver sections revealed a mild degree of extramedullary hematopoiesis in the younger animals (day 6-7), however, we detected no significant pathologic alterations. Sections of the spleen, thymus, lymph nodes, small intestine, esophagus and lungs revealed no pathologic alterations.

2.4.4 Increased serum IFN-γ and LPS sensitivity in tc-ptp$^{-/-}$ mice.

Our *in vivo* and *in vitro* results showing elevated IFN-γ production led us to examine the *in vivo* response to LPS and IFN-γ production in *tc-ptp*$^{-/-}$ animals. Twenty-day-old mice were injected i.p. with 4 μg LPS and kept under constant surveillance until their blood was collected five hours post-injection. Within an hour of injection, the majority of *tc-ptp*$^{-/-}$ mice were immobile. By four hours, they had developed symptoms of septic shock, including diarrhea, piloerection and a hunched posture, and their eyes were closed. None of the normal littermates or PBS-injected control mice showed similar signs or symptoms, even up to six hours after a higher dose of LPS (8 μg). Significant levels of serum IFN-γ were detected in PBS-injected *tc-ptp*$^{-/-}$ mice (Fig. 2.7). These levels were further increased by up to four-fold following LPS injection and reached levels that were ten-fold higher than those detected in age-matched LPS-injected *tc-ptp*$^{+/+}$ mice (Fig. 2.7). Administration of LPS did not significantly increase mean serum IFN-γ levels in *tc-ptp*$^{+/+}$ (Fig. 2.7) or *tc-ptp*$^{+/-}$ control mice even after administration of 8 μg LPS (data not shown).

Age	Organ	Frequency of mice with mononuclear infiltrate	
		No infiltrate	Mild to severe infiltrate
Day 6-7	Salivary gland	1/3	2/3
	Stomach	1/2	1/2
Day 13-14	Salivary gland	1/3	2/3
	Stomach	1/2	1/2
	Heart	0/2	2/2
Day 20-21	Salivary gland	0/3	3/3
	Stomach	0/3	3/3
	Liver	2/3	1/3
	Heart	0/3	3/3
	Kidney	1/3	2/3
	Skin	2/3	1/3

Table 2.1 – Age-dependent increase of inflammatory lesions in *tc-ptp*$^{-/-}$ mice.

Summary of pathology results in *tc-ptp*$^{-/-}$ mice at one, two and three weeks after birth. The *tc-ptp*$^{+/+}$ and *tc-ptp*$^{+/-}$ littermate controls showed no infiltrate.

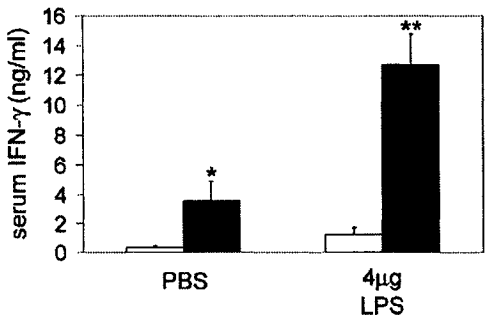


Figure 2.7 - Increased concentration of serum IFN-γ in tcptp-/- mice.

Serum IFN-γ in tcptp-/- (■) and tcptp+/+ mice (□) at three weeks after birth. Mice were injected with PBS or 4 μg LPS in 150 ml PBS and bled five hours post-injection. Results are shown as ng of IFN-γ/ml of mouse serum. Bars represent the mean values ± SE from four to eight mice. Statistical significance was determined by a two-tailed unpaired Student t test. * $P < 0.05$ tcptp-/- +LPS compared to tcptp-/- +PBS. ** $P < 0.01$ tcptp-/- +LPS compared to tcptp+/+ +LPS.

2.4.5 LPS sensitivity of tc-ptp$^{-/-}$ macrophages in vitro.

Evidence of excess IFN-γ *in vivo* led us to examine the activation status of *tc-ptp*$^{-/-}$ splenic macrophages. Cells from the macrophage/monocyte lineage are the only cell type that respond to IFN-γ and LPS by producing NO [278]. Furthermore, IFN-γ alone is able to induce some NO production in normal macrophages, but the cells do not respond to low concentrations of LPS unless previously primed by IFN-γ [268]. Both *tc-ptp*$^{+/+}$ and *tc-ptp*$^{-/-}$ splenocytes responded to a combination of IFN-γ and LPS by expressing iNOS mRNA (Fig. 2.8*a*) although in *tc-ptp*$^{-/-}$ cells upregulation was close to 10-fold greater, suggesting that they have a stronger maximum response, which could be due to prior exposure to and priming by IFN-γ.

When the cells were stimulated with various concentrations of LPS without exogenous IFN-γ and NO production was measured in supernatants after 48 hours, only *tc-ptp*$^{-/-}$ cells were able to respond to ng/ml concentrations of LPS (Fig. 2.8*b*, upper panel). Cells from 27-day-old *tc-ptp*$^{-/-}$ animals responded to a 1000-fold lower dose of LPS than those from day 14, and reached a plateau between 1 and 10 ng/ml LPS (Fig. 2.8*b*, lower panel). The maximum level of NO production was at least three-fold higher than for day 14 spleen cells. This demonstrates that *tc-ptp*$^{-/-}$ splenocytes have been primed by IFN-γ *in vivo*, and the priming effect increased with age, increasing both the sensitivity to LPS and the strength of the maximum response. To determine whether *tc-ptp*$^{-/-}$ cells were maximally stimulated by LPS alone, they were cultured with 10 ng/ml LPS and various concentrations of IFN-γ (10-500 U/ml). The maximum NO production under these conditions equaled the one reached in the absence of IFN-γ (21.7 ± 1.1 μM for *tc-ptp*$^{-/-}$ cells). The low NO produced by *tc-ptp*$^{+/+}$ cells (1.35 ± 0.2 μM with 10 ng/ml LPS + 100 U/ml IFN-γ) reflects the fact that normal unprimed splenocytes respond poorly to inflammatory stimuli, in contrast to the *tc-ptp*$^{-/-}$ cells.

To dissociate between the priming effect and an inherent sensitivity to LPS, spleen-derived macrophages grown from seven-day cultures were stimulated with various concentrations of LPS without exogenous IFN-γ and NO production was measured. Similar to the results obtained with whole spleen, only *tc-ptp*$^{-/-}$ macrophages were able to respond to pg/ml concentrations of LPS (Fig. 2.8*c*). This demonstrates that *tc-ptp*$^{-/-}$

splenic macrophages are inherently sensitive to low doses of LPS, as nearly all the cells were *de novo* derived *in vitro* and any priming effect due to prior exposure to IFN-γ would have disappeared [344]. No endogenous IFN-γ production was detected under these conditions (<30 pg/ml for both *tc-ptp*$^{-/-}$ and *tc-ptp*$^{+/+}$ cultures). Although the maximum NO production in response to a combined IFN-γ and LPS stimulus under these conditions was slightly higher in *tc-ptp*$^{-/-}$ than *tc-ptp*$^{+/+}$ cells (97.0 ± 7.7 μM vs. 76.5 ± 4.9 μM, respectively), it most likely reflects their increased responsiveness to IFN-γ [131]. We observed no difference in the number of live cells (as determined by MTT assay), cell morphology (size or granularity by flow cytometry), Gr-1 (Ly-6G) and Mac-1 (CD11b) staining or level of CD14 expression (data not shown), demonstrating that there was no apparent disparity in macrophage activation or development.

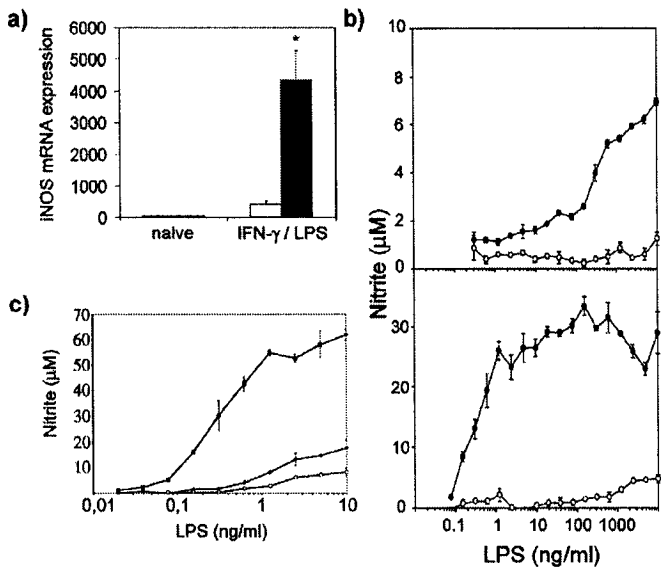


Figure 2.8 - Splenic macrophages from tcptp-/- mice display an inflammatory phenotype as shown by increased iNOS and NO in response to LPS.

A) iNOS mRNA levels in spleen cells from two-week-old tcptp-/- (■) and tcptp+/+ (□) mice after a stimulation with 100 U/ml IFN-γ and 10 ng/ml LPS for four hours. Expression level in naïve cells was set as one. Bars represent the mean values ± SE from five mice. Statistical significance was determined by a two-tailed unpaired Student t test. * $P < 0.05$ tcptp-/- compared to tcptp+/+. ***B)*** NO production as measured by nitrite concentrations in the supernatants from spleen cells from 14-day-old (upper panel) and 27-day-old (lower panel) tcptp -/- (●) and tcptp+/+ (○) mice cultured with different concentrations of LPS. Five separate experiments were performed and all gave similar results. The results shown are from one representative experiment. ***C)*** NO production as measured by nitrite concentrations in the supernatants from spleen-derived macrophages grown from 19-day-old tcptp-/- (■), tcptp+/- (●) and tcptp+/+ (○) mice and stimulated with different concentrations of LPS. Three separate experiments were performed and all gave similar results. The results shown are from one representative experiment.

2.5 DISCUSSION

The results presented here demonstrate that within days after birth *tc-ptp*$^{-/-}$ mice develop a systemic inflammatory disease. They are born at normal Mendelian ratios, indicating that the loss of TC-PTP is not lethal during embryonic development. There are no defects in embryonic organ development as shown by normal tissue architecture on day 6. However, we show progressive mononuclear infiltrates in non-lymphoid organs, accompanied by active cytokine production in the liver and salivary gland. The increase in cytokine expression together with the mononuclear infiltrates causes tissue damage and ultimately the death of the animal. Moreover, *tc-ptp*$^{-/-}$ mice show a significantly increased *in vivo* sensitivity to exogenous LPS and develop symptoms of endotoxic shock. These findings indicate that TC-PTP is a necessary component in the regulation of inflammatory responses and that its absence results in systemic inflammatory disease.

Susceptibility to endotoxic shock has been reported in inflammatory models [286, 351-353], and it has been shown to be dependent on IFN-γ and TNF-α production. Both cytokines are upregulated in *tc-ptp*$^{-/-}$ mice without exogenous stimulation and IFN-γ is strongly induced in response to LPS injection. LPS is constantly produced by resident enteric bacteria, but in healthy animals the liver detoxifies the small amount of LPS that crosses the gut epithelium and enters the portal circulation [354]. Injury to the liver or intestine may result in further LPS spilling over into the systemic circulation and stimulating primed macrophages to produce TNF-α and other cytokines in peripheral organs [286, 345]. The earliest cytokine upregulation in *tc-ptp*$^{-/-}$ mice is seen in the liver on day 3, which is likely to correspond to the activation of Kupffer cells that can also be primed to respond to LPS. The proliferation defect reported in *tc-ptp*$^{-/-}$ cells [3] could lead to an increase in gut permeability due to an inadequate turnover of intestinal epithelium. We also detected liver injury in older animals, at the time that the mice spontaneously develop symptoms of septic shock, such as diarrhea, runting and hunched posture. Endogenous LPS translocating from the gastrointestinal tract may therefore play an important role in the pathogenesis of the *tc-ptp*$^{-/-}$ phenotype, similar to what has been previously shown in GvHD [286, 345, 355, 356].

It was recently shown that the characteristic injury to epithelial tissues that occurs during the systemic inflammatory response that accompanies acute GvHD is not antigen-

restricted [357]. Similarly, widespread infiltrates seen in *tc-ptp*$^{-/-}$ mice also argue against an antigen-dependent mechanism and the pathology is more likely to be dependent on inflammatory cytokines as well as activation of components of the innate immune system. Splenic macrophages from *tc-ptp*$^{-/-}$ animals respond to very low concentrations of LPS alone while the same cells from normal animals require additional prior stimulation by IFN-γ in order to produce NO in response to LPS [266, 268]. The increased sensitivity to LPS *in vivo* suggests that the *tc-ptp*$^{-/-}$ macrophages are capable of becoming activated in response to very low levels of stimulus due to a combination of *in vivo* exposure to IFN-γ and inherent hypersensitivity and migrate into epithelial tissues, where they actively produce inflammatory mediators.

The priming effect observed in the absence of TC-PTP could be attributed to two independent factors. Firstly, we have shown increased IFN-γ production *in vivo*, hence, increased levels of the priming stimulus. Secondly, recent findings implicate TC-PTP in the control of Jak and Stat dephosphorylation downstream of cytokine receptors [128-131]. Bone marrow derived macrophages from *tc-ptp*$^{-/-}$ mice have increased Jak1 phosphorylation levels after stimulation with IFN-γ *in vitro* [131]. TC-PTP has also been identified as the phosphatase for Stat1 in fibroblast nuclei [128]. Since these kinases and transcription factors require phosphorylation to be active [11, 35], a lack of dephosphorylation in the *tc-ptp*$^{-/-}$ mice could result in constitutive signaling and lead to an exaggerated response, such as was seen with TNF-α and iNOS production. Both factors are likely to be important in the development of the *tc-ptp*$^{-/-}$ phenotype (Fig. 2.9).

The source of IFN-γ production by the *tc-ptp*$^{-/-}$ animals *in vivo* is yet unclear. Macrophages respond to LPS by producing IL-12 and IL-18, two cytokines that synergistically activate IFN-γ production by T cells and NK(T) cells [350, 358]. We show elevated IL-12 p40 expression in young *tc-ptp*$^{-/-}$ animals and their splenic T cells produce high amounts of IFN-γ *in vitro* in response to mitogenic stimuli despite lacking a proliferative response; however, NKT cells may be the cell population that most readily produces IFN-γ *in vivo* in response to LPS-induced IL-18 and IL-12 [359]. Cytokine-induced IFN-γ production in spleen cultures has also been attributed to NK1.1+ cells rather than T cells [360]. Despite the increased IFN-γ production shown here, treatment with anti-IFN-γ does not significantly increase the life expectancy of *tc-ptp*$^{-/-}$ mice (data

not shown), in contrast to what has been reported for the *socs-1*$^{-/-}$ mice [158]. This may reflect the different roles played by SOCS-1 and TC-PTP; the latter deactivates an active pathway, whereas the former inhibits the activation of the Jak/Stat pathway through binding the activation loop on Jaks [150]. Furthermore, while SOCS-1 is inducible [361] by cytokines, TC-PTP levels are regulated in a cell cycle dependent manner [362]. We are currently breeding our *tc-ptp*$^{-/-}$ mice onto an *ifng*$^{-/-}$ background to further study the mechanisms of inflammatory activation in the absence of TC-PTP.

The data presented here agree with previous studies from our laboratory [2] showing that the heterozygous *tc-ptp*$^{+/-}$ animals have no distinguishable phenotype. This is important because PTP-1B, a closely related phosphatase to TC-PTP, is now seen as a major target in the treatment of diabetes and obesity [4, 257]. Despite the obviously very different roles of TC-PTP and PTP-1B, to date no small molecule inhibitors have been reported that significantly discriminate between the two. Therefore, the absence of a *tc-ptp*$^{+/-}$ phenotype suggests that PTP-1B inhibitors could be safely used for the treatment of diabetes and obesity without inducing inflammatory side effects due to the partial loss of TC-PTP activity.

In conclusion, our results strongly indicate that TC-PTP is an important negative regulator of inflammatory reactions. The systemic inflammation seen in *tc-ptp*$^{-/-}$ mice leads us to hypothesize that the absence of the enzymatic activity or decreased expression of TC-PTP may be operative in many chronic inflammatory conditions, including GVHD. This implies that the upregulation of TC-PTP could potentially be used to control inflammatory responses. Conversely, TC-PTP downregulation could stimulate immune function in conditions where an augmented response may be beneficial, for example, the elimination of pathogenic organisms or in the treatment of neoplastic disease.

2.6 ACKNOWLEDGEMENTS

We thank Rosemary Siegrist-Johnstone and Ailsa Lee Loy for their excellent technical assistance.

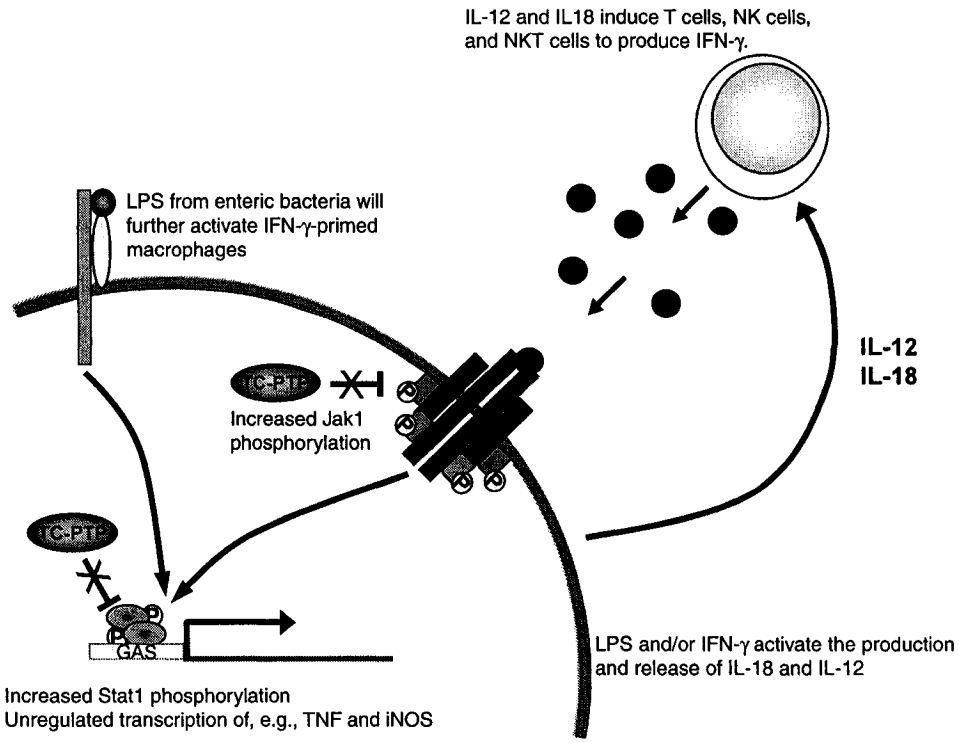


Figure 2.9 - Upregulation of the IFN-γ pathway in the absence of TC-PTP.

The loss of TC-PTP results in increased activation of the IFN-γ pathway through hyperphosphorylation of Jak1 [127] and Stat1 [124]. Stat1 activity induces TNF-α and iNOS expression. LPS increases IL-18 production which together with IL-12 serves as a positive feedback to IFN-γ producing cells. In the absence of TC-PTP this cycle cannot be turned off and results in the accumulation of IFN-γ, TNF-α, NO and activated inflammatory cells.

3 CHAPTER III – PROTEIN TYROSINE PHOSPHATASE 1B NEGATIVELY REGULATES MACROPHAGE DEVELOPMENT THROUGH CSF-1 SIGNALING

Preface

PTP-1B is the closest relative of TC-PTP in the PTP family. Although it has been reported to regulate Jak/Stat signalling, no immune phenotype had been reported. We were interested in studying macrophage development in PTP-1B deficient mice due to the significant effect that Jak2, a PTP-1B substrate, has in normal and defective myelopoiesis.

3.1 ABSTRACT

Protein tyrosine phosphatase 1B (PTP-1B) is a ubiquitously expressed cytosolic phosphatase with the ability to dephosphorylate JAK2 and TYK2, and thereby downregulate cytokine receptor signaling. Furthermore, PTP-1B levels are upregulated in certain chronic myelogenous leukemia patients, which points to a potential role for PTP-1B in myeloid development. The results presented here show that the absence of PTP-1B affects murine myelopoiesis by modifying the ratio of monocytes to granulocytes *in vivo*. This bias towards monocytic development is at least in part due to a decreased threshold of response to CSF-1, as the PTP-1B -/- bone marrow presents no abnormalities at the granulocyte-monocyte progenitor level but produces significantly more monocytic colonies in the presence of CSF-1. This is not due to an increase in receptor levels but rather enhanced phosphorylation of the activation loop tyrosine. PTP-1B -/- cells display increased inflammatory activity *in vitro* and *in vivo* through the constitutive upregulation of activation markers as well as increased sensitivity to endotoxin. Collectively, our data indicate that PTP-1B is an important modulator of myeloid differentiation and macrophage activation *in vivo*, and provide a first demonstration of a physiological role for PTP-1B in immune regulation.

3.2 INTRODUCTION

Protein tyrosine phosphatase 1B (PTP-1B; also known as PTPN1) is a ubiquitously expressed cytosolic phosphatase that is localized to the endoplasmic reticulum [236]. PTP-1B -/- mice were shown to be resistant to diet-induced diabetes and obesity [4, 125, 245, 246], and the enzyme has been implicated in several metabolic pathways due to its ability to dephosphorylate and thereby downregulate the activity of the insulin receptor [4, 245], epidermal growth factor receptor and platelet-derived growth factor receptor [241-243], insulin-like growth factor type I receptor [246, 247], as well as Jaks associated with cytokine family receptors, including leptin [5, 125] and growth hormone receptors [6]. PTP-1B appears to show specificity toward Jak2 and Tyk2 [7], and has been shown to regulate interferon signaling in fibroblasts [7]. Despite the regulatory role in cytokine signaling, no immunological phenotype has been attributed to the PTP-1B deficient mice to date.

IL-3, G-CSF and GM-CSF are cytokines that depend on Jak2 activity for signaling [363-365] and play important roles in the regulation of myeloid development [303]. The receptor for another important myeloid cytokine, CSF-1, belongs to the Class III receptor tyrosine kinase family together with the platelet derived growth factor receptor [14], and it also activates Tyk2 [366]. Furthermore, it has been shown previously that PTP-1B protein levels are upregulated in cell lines derived from chronic myelogenous leukemia (CML) patients [252, 326, 367]. These observations led us to investigate the role of PTP-1B in hematopoiesis and in particular, myelopoiesis.

The data presented here show that in the absence of PTP-1B, the monocyte to granulocyte ratio is affected *in vivo*. This is likely due to an increased sensitivity to CSF-1 as PTP-1B -/- bone marrow produces more colonies in response to CSF-1 and the receptor was hyperphosphorylated in response to CSF-1 in PTP-1B -/- bone marrow derived macrophages or in macrophages expressing the PTP1B DA substrate-trapping mutant. In addition, PTP-1B deficient macrophages display increased inflammatory phenotype *in vitro* and *in vivo* through the upregulation of activation markers as well as increased sensitivity to LPS. Together, our results demonstrate for the first time that PTP-1B regulates cytokine signaling in the immune system and thereby modulates myelopoiesis and macrophage activation *in vivo*.

3.3 MATERIALS AND METHODS

Mice. The generation of *ptpn1* $^{-/-}$ mice has been described previously [4]. The mice were kept in specific pathogen free housing and used between 6 and 15 weeks of age as specified. All animal work was carried out in accordance with the regulations of the Canadian Council of Animal Care and approved by the McGill Animal Care Committee.

Reagents, antibodies, and media. Cell culture reagents were purchased from Invitrogen (Burlington, ON, Canada) unless otherwise specified. LPS (*Escherichia coli* O111:B4) was obtained from Calbiochem (San Diego, CA), and prepared as previously described [286]. Serum for liquid cultures was endotoxin-free and purchased from Hyclone (Logan, UT). Recombinant murine (rm) IFN-γ was purchased from Invitrogen and rm CSF-1, rm G-CSF and rm GM-CSF from Stem Cell Technologies (Vancouver, BC, Canada). Antibodies for flow cytometry were purchased from BD Biosciences (Mississauga, ON, Canada), Serotec (Raleigh, NC), Biosource (Camarillo, CA) and Biolegend (Vineland, ON, Canada). Antibodies for western blotting were obtained from Cell Signaling (Pickering, ON, Canada), Upstate Biotechnology (Lake Placid, NY), and Santa Cruz Biotechnology (Santa Cruz, CA).

Flow cytometry. Fresh cell suspensions from bone marrow or spleen of age-matched male PTP-1B +/+ and -/- mice were prepared in PBS + 2% FBS. Non-specific binding was blocked with purified anti-CD16/CD32 antibody (BD Biosciences), and cells labeled with a combination of fluorochrome-conjugated substrate-specific antibodies. Data acquisition and analysis was conducted on FACSCalibur (Becton-Dickinson) using CellQuest software.

Methylcellulose colony assays. All colony assays were performed on young adult male mice (6-7 weeks). M3434 (Stem Cell Technologies) was used for mixed colony assays, and colonies were scored after 14 days based on morphology according to the manufacturer's instructions. For single cytokine colony assays, cells were cultured in M3231 (Stem Cell Technologies) and either CSF-1, G-CSF or GM-CSF as indicated. Colonies were scored on day 11.

Proliferation assays. Bone marrow cells from 7-week-old male mice were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) in DMSO as per manufacturer's instructions. The cells were cultured for 5 days in IMDM

supplemented with 10% FBS and 10 ng/ml CSF-1. The CSF-1 supplemented media was refreshed on day 4. On day 5, adherent cells were collected by scraping and analyzed by flow cytometry. Mitotic index was calculated as previously described [368].

Generation of stable cell lines. Retroviral vectors for PTP-1B have been previously described [248]. Raw 264.7 cells were exposed to the virus for 24 h, and then selected using 140 μg/ml Hygromycin. Two independent cell lines were used for either PTP-1B wild-type or DA mutant.

Spleen cultures. Spleen-derived macrophages were obtained essentially as previously described [349] using medium supplemented with 30% L-929 conditioned medium and 10% FBS. The L-929 medium was replaced with unsupplemented medium for 24 hrs before stimulation of the adherent cells with IFN-γ and/or LPS. Macrophage lineage was confirmed by CD11b and CD14 stain. Measurement of NO production in supernatant was performed essentially as previously described [300].

Determining LPS sensitivity in vivo. 12-14-week-old male mice were injected i.v. with 200 μl sterile PBS containing 0 mg/kg, 0.5 mg/kg or 4 mg/kg LPS. Mice were followed for 7 days after injection and scored for symptoms (body weight, eyes, fur, mobility, posture, diarrhea) twice daily. Mice were sacrificed if they lost 20% of their body weight or became hunched and immobile. Blood samples for serum cytokine levels were collected by cardiac puncture 4 hours post-injection, and analyzed with OptEIA™ mouse IFN-γ and IL-12 (p70) ELISA kits (BD Biosciences).

Statistical analysis. All statistical differences were determined by a two-tailed, unpaired, Student t analysis unless otherwise specified.

3.4 RESULTS

3.4.1 Augmented monocytic but not granulocytic development in the absence of PTP-1B.

The mice deficient for PTP-1B are viable and fertile with no reported gross abnormalities [4, 245]. The reports that PTP-1B regulates cytokine signaling by dephosphorylating Jak2 and Tyk2 [5-7, 125] led us to hypothesize that hematopoiesis may be affected by the loss of PTP-1B. Of particular interest was myelopoiesis, both due to the importance of Jak2 [363, 365] and the dysregulation of PTP-1B expression in CML [252, 326].

When total bone marrow from young adult (6- to 7-week-old) mice was cultured in the presence of myeloid growth factors (IL-3, IL-6 and SCF), PTP-1B -/- cells differentiated into monocytic colonies in a larger proportion than the PTP-1B +/+ cells (Fig. 3.1*a*). In keeping with the equal numbers of CFU-GM, the size of the $CD34^{+}CD16/CD32^{+}$ GMP population was not affected by the loss of PTP-1B (Fig. 3.1*b*), suggesting that the enzyme modulates lineage decision below the GMP level. Progenitor cells were first defined as $Lin^{-}Sca1^{-}CD127^{-}CD117^{+}$ and then divided into GMP, common myeloid (CMP), and megakaryocyte-erythrocyte progenitors (MEP) based on their CD34 and CD16/CD32 expression [302, 369].

3.4.2 The loss of PTP-1B sensitizes cells to CSF-1 and results in increased CSF-1R phosphorylation in bone marrow derived macrophages.

The major cytokines involved in monocyte development and differentiation are CSF-1 and GM-CSF. In single cytokine assays, PTP-1B affected monocytic colony formation only in response to CSF-1 (Fig. 3.2*a*). The number of colonies produced in the absence of PTP-1B was approximately three times that obtained with PTP-1B +/+ cells. Conversely, there was no difference in the number of monocytic colonies produced by PTP-1B +/+ vs. -/- bone marrow in response to a range of concentrations of GM-CSF (0.1 ng/ml – 15 ng/ml), or in the number of granulocytic colonies in response to G-CSF. There was no significant colony formation in the presence of serum alone (data not shown).

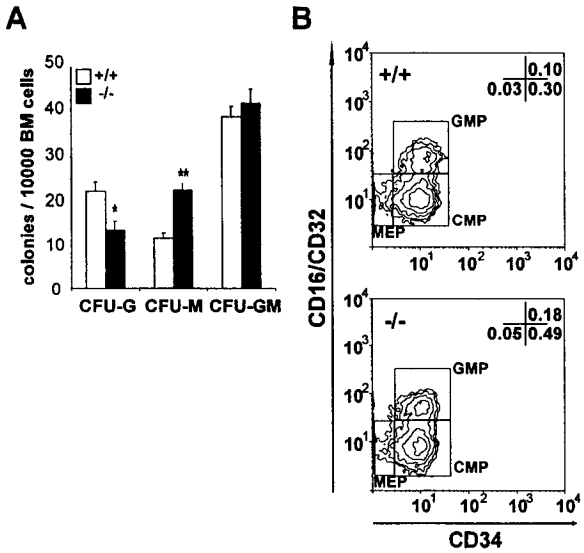


Figure 3.1 - Bias toward monocyte over granulocyte development in ptp1b-/- bone marrow.

A) Increased monocytic and decreased granulocytic colony development in a mixed colony assay using bone marrow from 6-week-old male mice. The histogram represents the mean ± SE from three separate experiments. n=8 for ptp1b+/+ and n=10 for ptp1b-/-. *p<0.01; **p<0.001. *B)* Representative FACS plots from 7-week-old males, demonstrating no significant changes in the myeloid precursor populations. Cells were first selected on Lin-Sca1-CD127-CD117+. Similar results were obtained in three separate experiments, with a total of 10 mice per genotype.

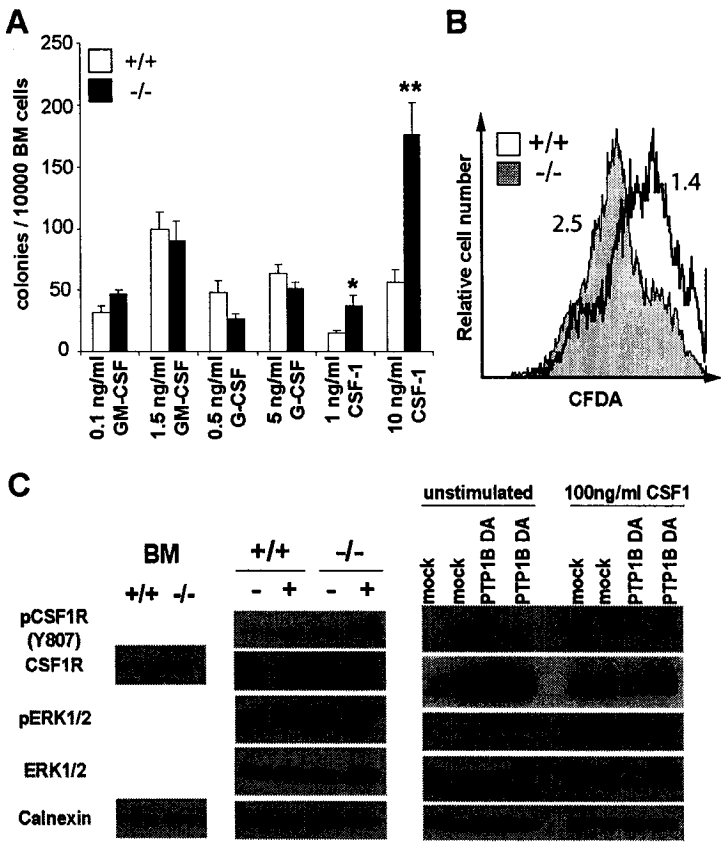


Figure 3.2 - The absence of PTP-1B results in increased responsiveness to CSF-1.

A) Colony assay with CSF-1, G-CSF or GM-CSF alone, demonstrating sensitivity to CSF-1 in -/- bone marrow. Data are presented as mean ± SE from three separate experiments. n=6-12 for +/+ and n=10-16 for -/-. *$p<0.05$; **$p<0.001$. *B)* Representative FACS plot demonstrating enhanced proliferation of bone marrow precursors in response to 10 ng/ml CSF-1. Cells were gated on CD11b+Ly6Glo. Mitotic indexes are shown next to the graphs. *C)* Left panel: Western blot showing equal CSF1R expression in +/+ and -/- total bone marrow. Middle panel: Increased CSF1R phosphorylation in -/- bone marrow-derived macrophages after a 1-minute stimulation with 10ng/ml CSF-1 at room temperature. Similar results were obtained in three independent experiments. Right panel: Expression of PTP-1B DA mutant increased CSF1R phosphorylation after a 1-minute stimulation with 100ng/ml CSF-1 at room temperature. Similar results were obtained with two independent cell lines.

The increased number of colonies pointed to an increased prevalence of CSF-1-responsive cells in the PTP-1B -/- bone marrow. The colonies also contained more cells (51 ± 10 cells / colony for PTP-1B +/+ vs. 110 ± 13 for PTP-1B -/-), indicating that the loss of PTP-1B may also increase proliferation of monocytic precursors in response to CSF-1. To verify this, we used CFSE to trace cell divisions and noted that the PTP-1B -/- cells divided on average one more time than the control cells over the 5-day incubation period as seen by a decrease in fluorescence due to cell division (Fig. 3.2*b*) and increased mitotic index (1.4 ± 0.2 for PTP-1B +/+ vs. 2.5 ± 0.6 for PTP-1B -/-; $p<0.05$) [368].

The increased sensitivity to CSF-1 was not due to an increase in receptor levels in total bone marrow (Fig. 3.2*c*, left panel) or in bone marrow depleted of erythrocytes ($Ter119^+$) and B lineage cells ($CD19^+$). To study CSF-1R phosphorylation, we stimulated bone marrow-derived macrophages with 10ng/ml CSF-1 at room temperature to minimize receptor internalization and thus optimize the detection of receptor phosphorylation. Under these conditions, the loss of PTP-1B resulted in hyperphosphorylation of the receptor on the activation loop tyrosine 807 (Fig. 3.2*c*, middle panel), indicating that PTP-1B indeed controls CSF-1R signaling at the level of receptor phosphorylation. To confirm these data, we created macrophage cell lines that stably expressed either wild-type or mutant PTP-1B. The expression of wild-type PTP-1B resulted in decreased receptor expression (Fig. 3.5), whereas the substrate-trapping mutant enhanced CSF-1R phosphorylation on tyrosine 807 (Fig. 3.2*c*, right panel) in two separate clones.

3.4.3 Increase in monocyte to granulocyte ratio in the periphery in PTP-1B -/- mice.

To investigate whether the bias in myelopoiesis was reflected in the periphery, we performed flow cytometry analysis on the spleen. Both monocytic and granulocytic populations were expanded (by 58% and 48%, respectively) in young adult PTP-1B -/- mice (Fig. 3.3*a*). We had detected no significant increase in bone marrow myelocytes in these same mice (data not shown). Therefore, the expansion of the splenic myelocytes could be due to either an increase in lifespan or to *in situ* hematopoiesis. In the case of

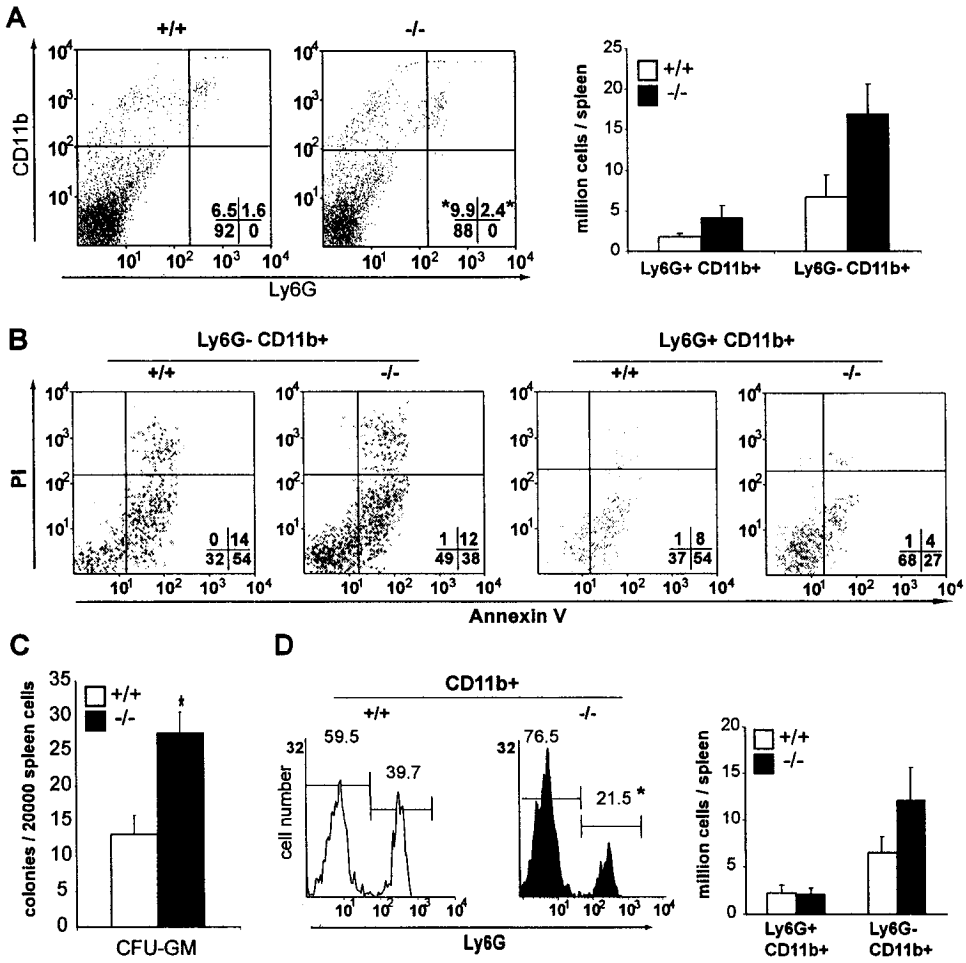


Figure 3.3 - Increase in peripheral CD11b+Ly6G- cells (monocyte/macrophages) in the ptp1b-/- mice.

A) Representative FACS plots from 6-week-old males, demonstrating an increase in both monocytes and granulocytes in the spleen. Histogram represents the mean ± SE of total number of cells per spleen. n=9 for ptp1b+/+ and n=13 for ptp1b-/- in four separate experiments. ***B)*** Representative FACS plots from 6-week-old males, demonstrating reduced apoptosis of both Ly6G-/lo monocytes and Ly6G+ granulocytes in the absence of PTP-1B. n=10 for both +/+ and -/- in three separate experiments. *p<0.002. ***C)*** Extramedullary myelopoiesis in the spleen of 6-week-old ptp1b-/- males as shown by mixed colony-forming assay. The histogram represents the mean ± SE from two separate experiments. n=6 for both +/+ and ptp1b-/-. *p<0.01. ***D)*** Representative FACS plots from 14-week-old males, demonstrating a switch in macrophage / granulocyte ratio. Histogram represents the mean ± SE of total number of cells per spleen. n=8 for both ptp1b+/+ and ptp1b-/- in two independent experiments.

PTP-1B -/- mice, both mechanisms appeared to play a role. Both monocytes and granulocytes exhibited reduced apoptosis as shown by decreased Annexin V staining (Fig. 3.3*b*). A mixed colony assay yielded two-fold more CFU-GM in the absence of PTP-1B, suggestive of an increase in splenic myeloid progenitor cells (Fig. 3.3*c*).

In older animals, the relative size of the granulocytic population was diminished in the absence of PTP-1B, reflecting the bias in myelopoiesis in favor of monocytes (Fig. 3.3*d*). Moreover, the expression levels of both CD11b and Ly6G on the surface of positive cells were decreased as seen by diminished mean fluorescence (Ly6G 343.3 ± 15.7 for +/+ and 308.0 ± 5.5 for -/-, $p<0.005$; CD11b 409.8 ± 102.9 for +/+ and 290.3 ± 13.8 for -/-, $p=0.06$). However, due to the increase in spleen cellularity (data not shown) in PTP-1B -/- mice, the absolute number of granulocytes was not diminished when compared to +/+ mice.

3.4.4 PTP-1B deficiency leads to an enhanced LPS sensitivity.

Macrophages normally need priming by, e.g., IFN-γ prior to an LPS stimulus for full activation and NO production [265, 268]. CSF-1, but not GM-CSF, can substitute for IFN-γ to some extent *in vitro* [370, 371], and the differences in CSF-1 sensitivity in PTP-1B -/- bone marrow cells led us to hypothesize that the loss of PTP-1B may also lead to an increased sensitivity to LPS. Using spleen-derived macrophages, we observed that the PTP-1B -/- cells were indeed more sensitive to low (picograms/milliliter) concentrations of LPS (Fig. 3.4*a*). There was no significant difference in NO production in response to IFN-γ alone (3.5 ± 1.6 μM for +/+ vs 8.2 ± 2.5 μM for -/-), nor when the cells were maximally activated with IFN-γ + LPS (37.2 ± 15.9 μM for +/+ vs 57.3 ± 12.0 μM for -/-). Spontaneous NO production was below detection limit (1-2 μM) for both PTP-1B +/+ and PTP-1B -/-. The increase in NO production was due to an upregulation of inducible nitric oxide synthase (iNOS) expression (Fig. 3.4*b*) despite no differences in JNK or p38 phosphorylation (data not shown). In accordance with Myers et al. [7], STAT1 phosphorylation was also enhanced downstream of IFN-γ (Fig. 3.4*c*). We did not detect any significant differences in baseline iNOS mRNA (data not shown). The level of CD14 or CD11b expression was not affected by the loss of PTP-1B (data not shown).

The loss of PTP-1B resulted in increased macrophage activation *in vivo* as well, as shown by the expansion of the $CD80^{+}Ly6G^{-}CD11b^{+}$ population in the spleen (Fig. 3.4*d*). The mean fluorescence for CD80 on positive cells was also increased (36.8 ± 4.1 for +/+ vs 55.8 ± 6.4 for -/-; $p<0.001$) indicating an upregulation of CD80 surface expression on individual cells. This was specific for CD80 as CD86 was not enhanced either in terms of surface expression levels nor the percentage of expressing cells (data not shown).

To demonstrate that the macrophage activation in PTP-1B -/- mice resulted in increased LPS sensitivity *in vivo* as well, three-month-old male mice were injected with 0.5 mg/kg LPS intravenously and bled two or four hours after injection. In PBS-injected control animals, there was no detectable IL-12 or IFN-γ (Fig. 3.4*e*). After two hours, IL-12 could be detected in most PTP-1B -/- animals and some +/+ controls, but it was significantly more strongly induced in the absence of PTP-1B (Fig. 3.4*e*, upper panel). Accordingly, IFN-γ, which was detectable after four hours, was also present at five-fold higher levels in PTP-1B -/- mice (Fig. 3.4*e*, lower panel). These data indicate that the increased activation of macrophages can be translated into an increased sensitivity to LPS *in vivo*.

The cytokine production was not only transient but developed into acute shock in the absence of PTP-1B. Mice injected with 4 mg/kg LPS were followed for seven days for symptoms of septic shock and sacrificed when they became severely hunched, when their mobility was decreased or if they lost more than 20% of their body weight. The majority of PTP-1B -/- mice became immobile and were sacrificed within 48 hours (Fig. 3.4*f*). None of these lost more than 10% of their weight. In comparison, only 20% control mice were sacrificed over the seven-day course of study and half of these were due to weight loss. Overall, weight loss was delayed by the absence of PTP-1B, possibly due to increased leptin signaling [372]. The mean symptom score was higher in PTP-1B -/- mice than PTP-1B +/+ controls throughout the course of study.

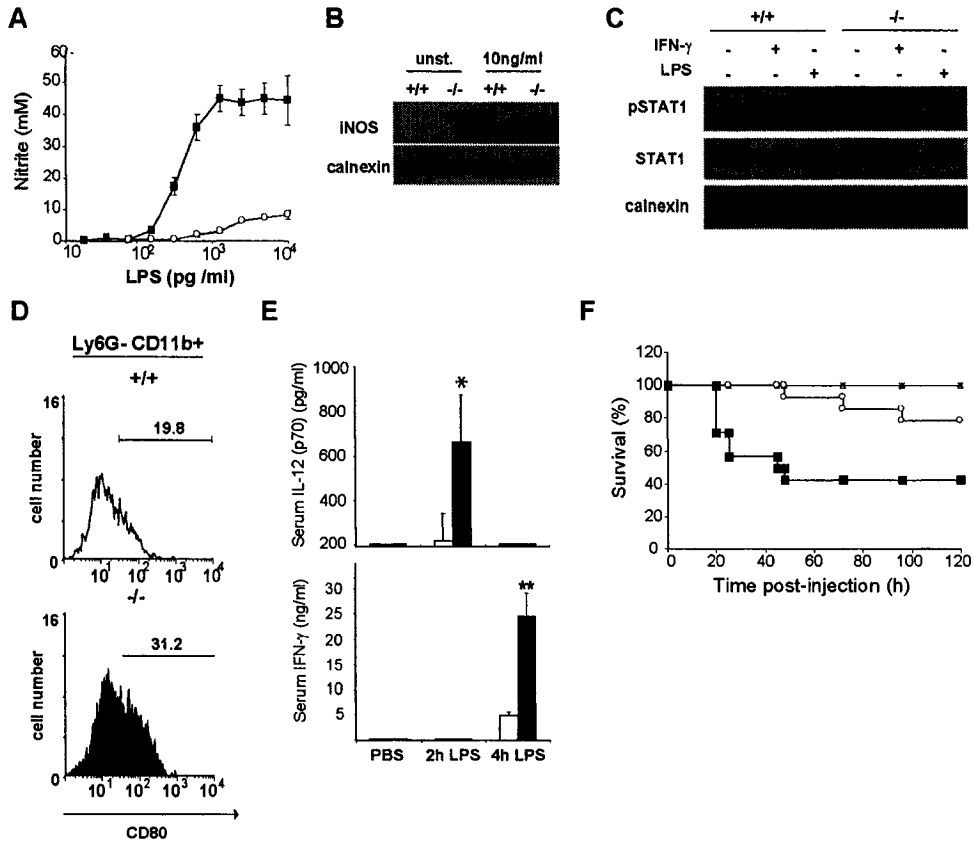


Figure 3.4 - Hypersensitivity to LPS in the absence of PTP-1B.

A) Highly increased LPS sensitivity as demonstrated by strong NO production by -/- (■) as compared to +/+ (○) spleen-derived macrophages. The results are representative of three separate experiments with a total of 6 mice per group. *B)* Increase in iNOS expression in response to 10 ng/ml LPS in -/- as compared to +/+ cells. Similar results were obtained in three separate experiments. *C)* Increased STAT1 phosphorylation in response to 100 U/ml IFN-γ in the absence of PTP-1B. Similar results were obtained in three separate experiments. *D)* Representative FACS plots showing increased CD80 surface expression on CD11b+Ly6G-/lo cells in the absence of PTP-1B, expressed as average percentage of CD80+ cells from three independent experiments. n=12 for both +/+ and -/-. *E)* Increased serum IL-12 (p70) (upper panel) and IFN-γ (lower panel) two and four hours after an i.v. injection with 0.5 mg/kg LPS in the absence of PTP-1B. The data are presented as the mean ± SE from three separate experiments. n=7 for both groups at 2h; n=10 for both groups at 4h. * p=0.05 and **p<0.005 using the Wilcoxon rank sum test. *F)* The loss of PTP-1B decreases survival after an i.v. injection with 4mg/kg LPS. x=control (PBS-injected) PTP-1B +/+; Δ= control (PBS-injected) PTP-1B -/-; ○=LPS-injected PTP-1B +/+; ■ =LPS-injected PTP-1B -/-. n=9 for LPS-injected and n=4 for PBS-injected animals.

3.5 DISCUSSION

The data presented above identify PTP-1B as a critical regulator of macrophage and granulocyte development and lineage specification. The absence of PTP-1B in mice resulted in a significant increase in the monocyte / macrophage population in the spleen and bone marrow (data not shown) as the mice became older. This switch in differentiation was translated into an increased number of CSF-1 –responsive cells in the PTP-1B -/- bone marrow as well as a decreased threshold to both CSF-1 and LPS, due to hyperphosphorylation of the CSF-1R. Collectively, our results suggest that PTP-1B plays a role in lineage commitment after the common granulocyte-monocyte precursor stage.

The reason for the enhanced sensitivity of PTP-1B -/- bone marrow cells to CSF-1 appears to stem from the increased phosphorylation of the receptor upon stimulus at the level of the activation loop tyrosine. The overall level of receptor expression was not affected by the deletion of PTP-1B, although it is possible that the level of receptor on a subset of cells is different, similar to what we detected in cell lines overexpressing PTP-1B. It has been previously published that CSF-1R is not a substrate of PTP-1B in COS cells [241]. The authors looked at the total tyrosine phosphorylation of the receptor, however, whereas we concentrated on a specific residue. Moreover, modulation of CSF-1R signaling by PTP-1B may be restricted to a particular cell lineage. Due to its location in the endoplasmic reticulum and contact with the endosomal compartment, PTP-1B may also play an indirect role in receptor signaling by modulating trafficking and recycling. It was recently reported to modulate the maturation of Flt3, a receptor related to CSF-1R [373]. Given our overexpression results, it might be interesting to further study the role of PTP-1B in this context.

PTP-1B not only regulates the lineage specification but also activation of monocyte/macrophage lineage cells. Tissue macrophages in the absence of PTP-1B display an activated phenotype, with a significant percentage of them expressing CD80. Exclusive upregulation of CD80 but not CD86 has previously been reported in pulmonary alveolar macrophages [374, 375] from asthmatic patients as well as in spleen-derived dendritic cells [376] in a mouse model of allergic asthma. CD80 was proposed to play a role in the maintenance of inflammation, while CD86 was important for its induction. Together with the decreased level of CD11b expression on individual cells, the increase

in CD80 levels points to the development of a variant type of macrophages with a predisposition to an inflammatory type of immune response. Lower levels of CD11b on the monocyte surface are also an indication of a potentially decreased phagocytic capacity. It has been previously shown that integrin signaling is affected by the loss of PTP-1B [249, 251], which may have an impact on exocytosis and phagocytosis. We are currently investigating the role of PTP-1B in the clearance of intracellular pathogens.

CSF-1 stimulation is known to enhance LPS sensitivity [370, 371]. Although the loss of PTP-1B is not lethal in mice under normal, controlled conditions, challenge with LPS resulted in increased systemic IFN-γ production as well as decreased survival of PTP-1B -/- mice. This is most likely due to a decreased threshold of PTP-1B -/- macrophages to LPS, resulting in enhanced IL-12 secretion, which would in turn activate IFN-γ production by T cells and natural killer (NK) cells [350]. Recent findings indicate that PTP-1B deficiency confers protection from endoplasmic reticulum stress –induced cell death [248] and Fas-mediated liver damage [377]. PTP-1B -/- macrophages could therefore be more resistant to activation-induced apoptosis, and show prolonged potential for cytokine and nitrogen radical secretion. We have also detected an increase in the $DX5^{+}TCR\beta^{+}$ population in the spleen of PTP-1B -/- mice (K.M.H. and M.L.T., unpublished data), suggesting that part of the *in vivo* response to LPS may also be directly due to splenic NKT cells [359].

We did not detect any difference in the number of GMPs in the bone marrow, or any impairment in granulocyte survival, suggesting that the decrease in the percentage of splenic granulocytes may be directly due to the increased numbers of monocytes. Indeed, when one accounts for the increase in spleen cellularity, there is no actual decrease in the absolute number of granulocytes in PTP-1B -/- mice (2.3 ± 0.8 million for +/+ vs. 2.1 ± 0.6 million for -/-). The increased sensitivity of PTP-1B deficient cells to CSF-1 may in this setting play a role in the lineage commitment. Sustained expression of transcription factors, such as Pu.1 [313, 314, 378] and Early growth response 1 (Egr-1) [318] also favors monocytic differentiation. Egr-1 activity suppresses PTP-1B expression [379], and the loss of PTP-1B could in this context mimic sustained Egr-1 expression. The increased sensitivity to IFN-γ may also increase the activity of IFN regulatory factor-8 and thus modulate cell fate towards monocyte/macrophage lineage [319]. Interestingly, we did not

see increased colony formation by PTP-1B -/- bone marrow in response to either GM-CSF or G-CSF, both of which signal through Jak2, clearly indicating that PTP-1B controls myeloid lineage commitment independent of cytokine signaling in addition to its role downstream of CSF-1R.

PTP-1B inhibitors are currently under study as treatment modalities for obesity and type II diabetes [257, 258]. In our study, heterozygous animals did not show appreciable intermediate phenotype. Also, the development of increased LPS sensitivity most likely requires changes at the level of macrophage development, as short-term treatment with suramin, a broad-spectrum PTP inhibitor, was shown to be protective against liver damage due to endotoxic shock [380]. Therefore, although PTP-1B undoubtedly plays an important role in myeloid development and activation, the short term use of inhibitors would most likely not have deleterious effects on macrophage function in humans. Conversely, the results presented here identify PTP-1B as a potential target for treating myeloid malignancies, as the level of PTP-1B expression affects myeloid commitment. The increase in PTP-1B expression in CML patients has been documented [252, 326, 367], but the actual consequences of this increase are not known. It is therefore possible that decreasing the level of PTP-1B in these cells could influence their differentiation and interferon sensitivity, and consequently response to treatment [381].

In conclusion, our data indicate a novel role for PTP-1B in myeloid development and CSF-1 signaling in macrophages and identify it as a potential target for the treatment of myeloid malignancies.

3.6 ACKNOWLEDGEMENTS

We thank Rosemary Siegrist-Johnstone and Ailsa Lee Loy for technical assistance, Malcolm Baines and Sofi Julien for helpful comments on the manuscript, and Eric Massicotte and Martine Dupuis for their technical expertise on flow cytometry.

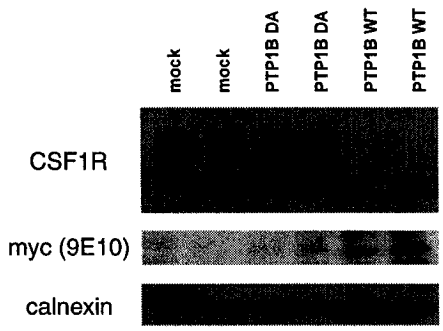


Figure 3.5 - Sustained PTP-1B expression decreases CSF-1R levels in Raw 264.7 cells.

Representative western blot showing decreased CSF-1R expression in cells expressing exogenous PTP-1B as compared to mock-infected or cells expressing the substrate-trapping mutant PTP-1B DA. Membrane was also blotted for myc to verify the expression of myc-tagged PTP-1B. Calnexin was used as loading control.

4 CHAPTER IV – COMPLEMENTARY ROLE FOR TC-PTP AND PTP-1B IN INTERFERON-GAMMA SIGNALING

Preface

Due to the high structural similarity and overlapping substrate specificity of TC-PTP and PTP-1B, we wished to study the impact of combined ablation of both phosphatases *in vivo*.

4.1 ABSTRACT

The control of tyrosine phosphorylation depends on the fine balance between kinase and phosphatase activities. Protein tyrosine phosphatase 1B (PTP-1B) and T cell protein tyrosine phosphatase (TC-PTP) are two closely related phosphatases that are known to control cytokine signaling, for example, in macrophages. We wished to study the redundancy of PTP-1B and TC-PTP on interferon signalling by deleting one or both copies of PTP-1B in *tcptp*$^{-/-}$ and *tcptp*$^{+/-}$ mice by interbreeding. Our results indicated that the double mutant was lethal at a relatively early stage of embryonic development. Mice heterozygous for TC-PTP on the *ptp1b*$^{-/-}$ background developed symptoms similar to a chronic inflammatory disease, and their macrophages were highly sensitive to interferon-γ as shown by increased Stat1 phosphorylation and nitric oxide production. Together, these data indicate a nonredundant role for PTP-1B and TC-PTP in the regulation of interferon signalling.

4.2 INTRODUCTION

T cell protein tyrosine phosphatase (TC-PTP; also known as PTPN2) and protein tyrosine phosphatase 1B (PTP-1B; also known as PTPN1) are two intracellular phosphatases with a high degree of sequence and structural homology within the catalytic domain [8, 328]. PTP-1B is known as a metabolic regulator protein due to its ability to dephosphorylate the insulin receptor [4, 245] and its association with Type II diabetes [262]. *ptp1b*$^{-/-}$ mice are protected from diet-induced insulin-resistance and obesity [4, 5, 125], a phenotype which has incited a lot of research from the part of pharmaceutical companies to develop PTP-1B inhibitors to treat type II diabetes [258]. However, due to the structural homology with TC-PTP, none of the small molecule inhibitors developed to date exhibit a high degree of selectivity against TC-PTP.

In addition to the role in insulin signalling, PTP-1B has also been shown to modulate cytokine receptor signalling [5-7, 125]. Recent data from our laboratory indicate that PTP-1B plays an important role in B lymphocyte [327] and macrophage development [382] (see chapter 3). TC-PTP is also known to affect hematopoiesis at several different levels [2, 301, 383]. One of the common pathways that both enzymes have been shown to regulate is interferon (IFN)-γ signalling [7, 131].

IFN-γ is a major inflammatory cytokine, which plays an important role in macrophage activation. The ligation of the IFN-γ receptor results in the activation of cytoplasmic Jak1 and Jak2 kinases as well as the transcription factor Stat1[12]. Jak1 and Stat1 are known substrates of TC-PTP [128, 131], whereas Jak2 is known to be dephosphorylated by PTP-1B[7]. We have previously shown that the IFN-γ pathway is hyperactive in *tcptp*$^{-/-}$ mice [301], and that *ptp1b*$^{-/-}$ macrophages are activated. We therefore hypothesized that the deletion of both TC-PTP and PTP-1B would exacerbate the inflammatory phenotype seen in *tcptp*$^{-/-}$ mice.

The data presented here indicate that the at least one copy of *tcptp* or *ptp1b* is necessary for normal embryonic development. In addition, TC-PTP and PTP-1B appear to have additive roles in macrophage activation and development as shown by increased colony formation in response to CSF-1 by *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ bone marrow precursors and enhanced sensitivity to IFN-γ of *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ spleen-derived macrophages. Lastly, our

results show that the ablation of PTP-1B in *tcptp*$^{+/-}$ mice reveals a thus far undetected gene dosage effect, and suggest that the use of small molecule inhibitors against PTP-1B that also inhibit TC-PTP may have beneficial side effects in further enhancing interferon sensitivity.

4.3 MATERIALS AND METHODS

Mice. ptp1b$^{-/-}$ mice were initially bred with *tcptp*$^{+/-}$ mice to obtain *tcptp*$^{+/-}$*ptp1b*$^{+/-}$ mice, which were then interbred to obtain all different genotypes. Genotyping was done as previously described [2, 4]. All mice were kept in specific pathogen free housing in the animal care facility. Protocols were approved by the McGill animal care ethics committee.

Reagents, antibodies, and media. Cell culture reagents were purchased from Invitrogen (Burlington, ON) unless otherwise specified. Antibodies for flow cytometry were purchased from BD Biosciences (Mississauga, ON, Canada), Serotec (Raleigh, NC), Biosource (Camarillo, CA) and Biolegend (Vineland, ON, Canada). Antibodies for western blotting were obtained from Cell Signaling (Pickering, ON, Canada), Upstate Biotechnology (Lake Placid, NY), and Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant murine interferon-γ was purchased from Invitrogen and ConcavalinA from Pharmacia (Baie-d'Urfé, QC). ^{3}H-thymidine for the proliferation assays came from ICN (Irvine, CA).

Histology. The submandibular and lacrimal glands were carefully removed from 4-6-month-old mice and placed in OCT compound. The fixed tissues were frozen, and sections were stained with haematoxylin-eosin (H & E). Stained tissues were examined by light microscopy without knowledge as to the genetic composition of the sample.

Flow cytometry. Fresh cell suspensions from thymus, axillary lymph nodes (LN) or spleen were prepared in PBS + 2% fetal bovine serum (FBS). Cells were incubated with purified anti-CD16/CD32 antibody (BD Biosciences) to block non-specific binding to Fc-receptors, followed by a combination of fluorochrome-conjugated substrate-specific

antibodies. Data acquisition and analysis was conducted on FACScan (Becton-Dickinson) using CellQuest software.

Methylcellulose colony assays. All colony assays were performed on young adult male mice (6-7 weeks). Total bone marrow cells were cultured in M3231 (Stem Cell Technologies) and either CSF-1 or GM-CSF as indicated. Colonies were scored on day 11.

Nitrite assay. Spleen-derived macrophages were obtained essentially as previously described [349]. In brief, spleen cells were cultured for seven days in 12-well plates at $1.6x10^6$ cells / well and in medium supplemented with 30% L-929 conditioned medium and 10% FBS. The supplemented medium was refreshed on day four. The L-929 medium was replaced with unsupplemented medium for 24 hrs before stimulation with IFN-γ. Measurement of NO production was performed essentially as described previously [300]. For signaling studies, spleen-derived macrophages were serum starved overnight, and then left unstimulated or stimulated with 100U/ml IFN-γ for 15 minutes.

Preparation of cell lysates and immunoblotting. Cells were washed in ice-cold PBS and lysed in radioimmunoprecipitation buffer supplemented with Complete EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals, Laval, QC, Canada) and phosphatase inhibitors (1mM Na_3VO_4 and 50mM NaF). Lysates were cleared by centrifugation and the protein content was measured by the Bradford method (Biorad, Missisauga, ON, Canada). Samples were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

Measurement of IFN-γ. Splenic T cells were purified by positive selection for CD90.2 using the EasySep™ magnetic nanoparticles (StemCell Technologies, Vancouver, BC). For IFN-γ production, total spleen cells or CD90.2 -selected T cells were cultured on plates coated with 5ug/ml anti-CD3 and 10ug/ml anti-CD28 for 72 hours. IFN-γ in the supernatant was measured with OptEIA™ mouse IFN-γ ELISA (BD Pharmingen). For proliferation assay, total spleen cells were grown for 48 hours in a 96-well plate at $5x10^5$ cells/well in medium supplemented with Hepes buffer and 2.5μg/ml ConA, after which ^{3}H-thymidine was added to the cultures for 16 hours and DNA synthesis was determined by measuring incorporated radioactivity on beta-counter.

4.4 RESULTS

4.4.1 The *tc-ptp*$^{-/-}$*ptp-1b*$^{-/-}$ double mutant is lethal during embryonic development.

Due to the overlapping substrate specificities of TC-PTP and PTP-1B as well as the high structural homology in their catalytic domains, we decided to obtain *tcptp*$^{-/-}$*ptp1b*$^{-/-}$ double deficient mice to study the redundancy of TC-PTP and PTP-1B function in vivo. Using *tcptp*$^{+/-}$*ptp1b*$^{+/-}$ mice as breeders, we genotyped the progeny immediately after birth (at 3-5 days of age) due to the early lethality of the *tcptp*$^{-/-}$ mice and obtained no double mutants (see table 4.1), suggesting that the loss of both TC-PTP and PTP-1B resulted in early embryonic death.

We began genotyping embryos soon after turning (8.5 days p.c.) and discovered that *tcptp*$^{-/-}$*ptp1b*$^{-/-}$ mice were viable until 9.5-10.5 days p.c. but none could be detected past 12 days p.c. (Fig. 4.1*a*). At E10.5, they were smaller than their littermates (Fig. 1*b*), but no overt phenotype was detected. It must be noted that the incidence of resorptions at 12 days p.c. was close to 15%, which is much more than the expected ratio of *tcptp*$^{-/-}$*ptp1b*$^{-/-}$ embryos. Also, the incidence of live *tcptp*$^{-/-}$ *ptp1b*$^{+/-}$ births was somewhat lower than expected (Table 4.1), suggesting that some of these mice may also die *in utero* and contribute to the resorption rate observed.

4.4.2 Haploinsufficiency of *tc-ptp* becomes apparent on *ptp1b*$^{-/-}$ background.

In previous reports, young *tcptp*$^{+/-}$ mice have not been shown to differ from *tcptp*$^{+/+}$ littermates, suggesting that one copy of *tcptp* is sufficient to maintain normal immune development and function [2, 301]. However, when put on *ptp1b*$^{-/-}$ background, it rapidly became evident that a single copy of *tcptp* was no longer sufficient. Although both *tcptp*$^{+/-}$ and *ptp1b*$^{-/-}$ mice have essentially normal life-spans and are fertile, more than half of *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ mice die by three months of age (Fig. 4.2*a*) and the remaining mice do not survive past 9 months (data not shown). They have lower body weight, and exhibit significant splenomegaly and lymphadenopathy. They also display symptoms of ocular infections (crusty, swollen eyes) at a high frequency, which could be due to inflammation [384-386], as we detect massive mononuclear infiltrates to the lacrimal (Fig. 4.2*b*) and

Genotype (tc-ptp ptp-1b)	Obtained ratio	Expected ratio
+/+ +/+	0.053	0.063
+/+ +/-	0.149	0.125
+/+ -/-	0.062	0.063
+/- +/+	0.166	0.125
+/- +/-	0.280	0.250
+/- -/-	0.135	0.125
-/- +/+	0.068	0.063
-/- +/-	0.087	0.125
-/- -/-	0	0.063

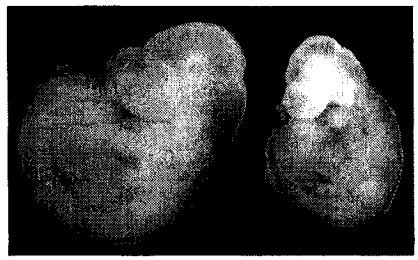
Table 4.1 – The distribution of genotypes in the progeny from heterozygous matings. Pups were identified and a piece of tail was obtained for genotyping at 3-5 days after birth. The observed frequencies are on the left and the expected Mendelian frequencies on the right. n=483. $\chi^2<1x10^{-6}$.

A)

frequency of tcptp -/-ptp1b-/- embr yos

	obtained	expected
E9.5-10.5	0.066	0.063
E11.5	0.022	0.063
E12.5	0	0.063

B)



tc+/-1b+/- tc-/-1b-/-

Figure 4.1 - Deficiency in TC-PTP and PTP-1B is lethal at E10.5-11.5.

A) A table showing the obtained and expected frequencies of tcptp-/-ptp1b-/- mice at E9.5 through E12.5. ***B)*** Photograph of E10.5 embryos.

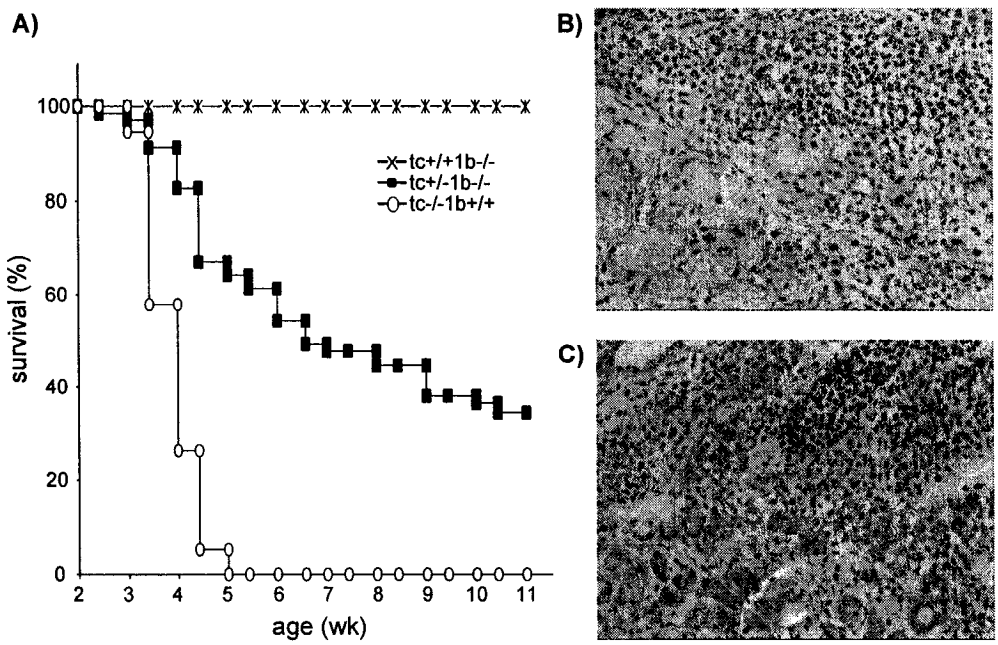


Figure 4.2 - Decreased survival of tcptp+/-ptp1b-/- mice

A) Survival graph showing that 60% tcptp+/-ptp1b-/- mice die within 2.5 months after birth. Photomicrograph of a ***B)*** lacrimal gland and a ***C)*** submandibular salivary gland of a 5-month-old tcptp+/-ptp1b-/- mouse.

salivary glands (Fig. 4.2*c*). Such pathology is associated with, e.g., Sjogren's syndrome in humans.

It is also noteworthy that *tcptp*$^{-/-}$ *ptp1b*$^{+/-}$ mice have a significantly shorter life-span than *tcptp*$^{-/-}$ *ptp1b*$^{+/+}$ mice (mean survival 20.4 ± 1.7 days for *tcptp*$^{-/-}$ *ptp1b*$^{+/-}$ versus 25.3 ± 3.5 days for *tcptp*$^{-/-}$ *ptp1b*$^{+/+}$; $p<0.0001$). Conversely, *tcptp*$^{+/-}$ *ptp1b*$^{+/-}$ mice are fertile and survive past 12 months.

4.4.3 TC-PTP and PTP-1B collaborate on macrophage development.

Given the appearance of *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ mice, which resembled to a certain extent that of *tcptp*$^{-/-}$ mice [2, 301], as well as the current evidence for both TC-PTP and PTP-1B in the control of IFN-γ signalling (see Chapter 3 and refs. [7, 131]), we hypothesized that *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ mice might exhibit defective macrophage development and activation. It has been previously shown that *tcptp*$^{-/-}$ mice have an increased number of F4-80^{+} cells in the spleen [2], and that *ptp1b*$^{-/-}$ mice have more CSF-1-responsive cells in the bone marrow as well as an increase in CD11b^{+}Ly6G$^{-/lo}$ cells in the spleen [382] (see Chapter 3). Therefore, we wished to look at CSF-1 responsiveness and splenic macrophages in *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ mice.

In the methylcellulose colony assay, the number of colonies reflects the number of cytokine-responsive precursors in the bone marrow. As expected, *tcptp*$^{+/-}$ *ptp1b*$^{+/+}$ bone marrow had the same number of macrophage precursors as normal bone marrow (Fig. 4.3*a*). There was also no difference in the number of precursors from *tcptp*$^{+/-}$ *ptp1b*$^{+/-}$ bone marrow, and the *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$ bone marrow produced expectedly 2.5 –fold more colonies than the control. Data from our laboratory suggests that TC-PTP also regulates CSF-1 signalling [383]. Therefore, it was not that surprising that *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ mice had even more CSF-1 responsive cells than *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$ mice (Fig. 4.3*a*). This was specific to CSF-1, as stimulation with GM-CSF did not produce any differences in the number of colonies independent of the genotype.

In terms of relative numbers of splenic macrophages, *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$ and *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ mice both had a larger percentage of cells that were CD11b^{+}Ly6G$^{-/lo}$ when compared to *tcptp*$^{+/+}$ *ptp1b*$^{+/+}$ controls (Fig. 4.3*b*). However, there was no significant difference between the two. Taken into account the significant splenomegaly in

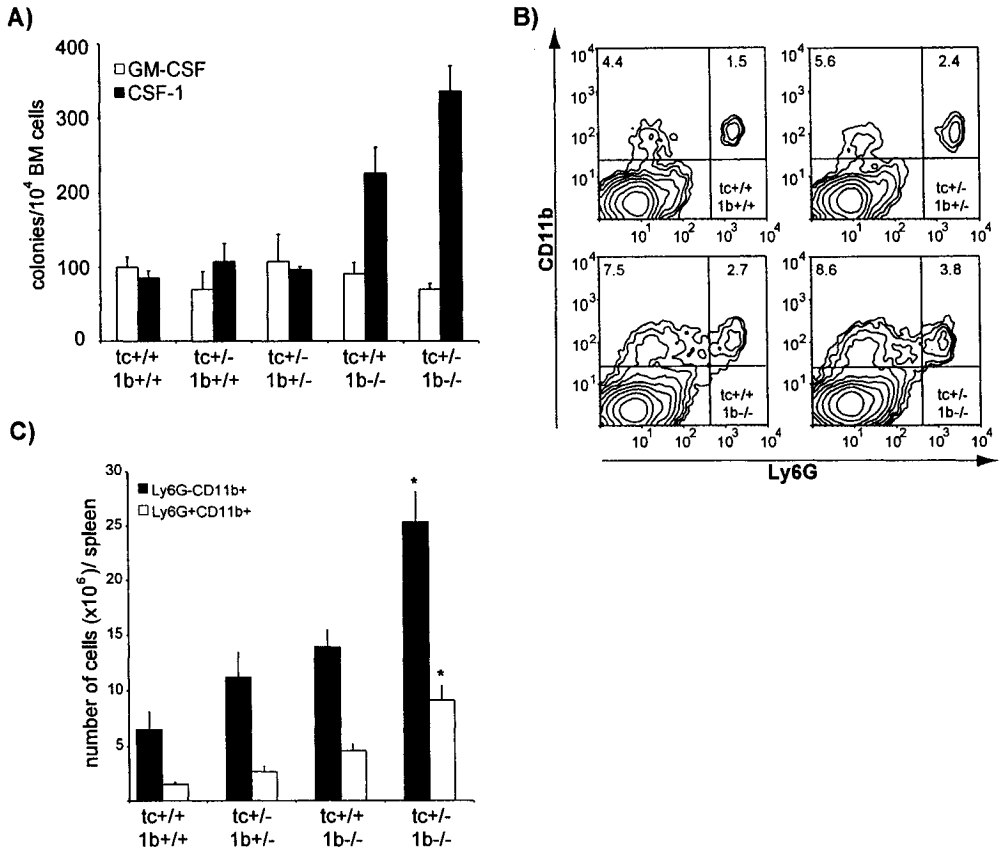


Figure 4.3 - TC-PTP and PTP-1B collaborate in macrophage development.

A) Methylcellulose colony assay showing an additive effect of the loss of TC-PTP and PTP-1B in the response to CSF-1. Bars represent mean ± SE from two separate experiments. n=4-6 for each genotype. ***B)*** Representative flow cytometry plots from spleen of 6-7-week-old mice. ***C)*** Absolute numbers of macrophages and granulocytes per spleen from 6-7-week-old mice. Bars represent mean ± SE from four separate experiments. n=5-9 for each genotype. * $p<0.01$ tcptp+/- ptp1b-/- compared to tcptp+/+ ptp1b-/-.

tcptp$^{+/-}$ *ptp1b*$^{-/-}$ animals ($256x10^6 \pm 28x10^6$ cells/spleen versus $171x10^6 \pm 14.2x10^6$ for *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$ and $103x10^6 \pm 10.6x10^6$ for *tcptp*$^{+/+}$ *ptp1b*$^{+/+}$; $p<0.02$ for both), the total number of macrophages in *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ spleen is approximately double of that in *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$ spleen and over three-times that in normal mice (Fig. 4.3*c*). These data show that TC-PTP and PTP-1B play a cooperative role in macrophage development. However, it seemed doubtful to us that the differences detected between *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$ mice and *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ mice would be sufficient to explain the death of the latter, when the former have a perfectly normal life-span.

4.4.4 Thymic defect in the *tcptp*$^{+/-}$*ptp1b*$^{-/-}$ mice.

Thus, we wished to investigate the T cell function in *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ mice. Previous data show that *tcptp*$^{-/-}$ mice have essentially normal numbers of peripheral T cells, which do not proliferate in response to mitogenic stimuli [2] but produce large amounts of IFN-γ [301]. There is no reported T cell phenotype in *ptp1b*$^{-/-}$ mice [327].

When looking at the thymus, we noticed a decrease in $CD4^+CD8^+$ double-positive thymocytes, which was accompanied by a relative increase of mature single-positive $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in the *tcptp*$^{+/-}$ *tcptp*$^{-/-}$ mice (Fig. 4.4*a*). When the immature lineage-negative (CD3-CD4-CD5-CD8-CD11b-CD19-DX5-Ter119-) cells were further divided based on the expression of CD44 and CD25, we could detect a partial blockage at the earliest DNI (CD44+CD25-) stage (Fig. 4.4*b*). However, as this thymic defect was accompanied by a significant decrease in total cellularity (see table 4.2), it may be secondary to stress and inflammation [387]. In this respect, we detected a significant proportion of CD11b+ cells in the *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ thymus (Fig. 4.4*c*), a population that was absent from both normal and *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$ thymi. It is possible that these infiltrating myeloid cells produce NO, which is highly toxic to double-positive thymocytes [279, 280] and thus could contribute to the thymic defect.

This hypothesis is further strengthened by the relatively mild differences observed in the T cell population in the periphery. Similar to what was published for *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$ mice [327], we saw an accumulation of $B220^+$ cells in the axillary LN of *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ mice (Fig. 4.5*a*). Due to the lymphadenopathy, this reflected a true increase in the

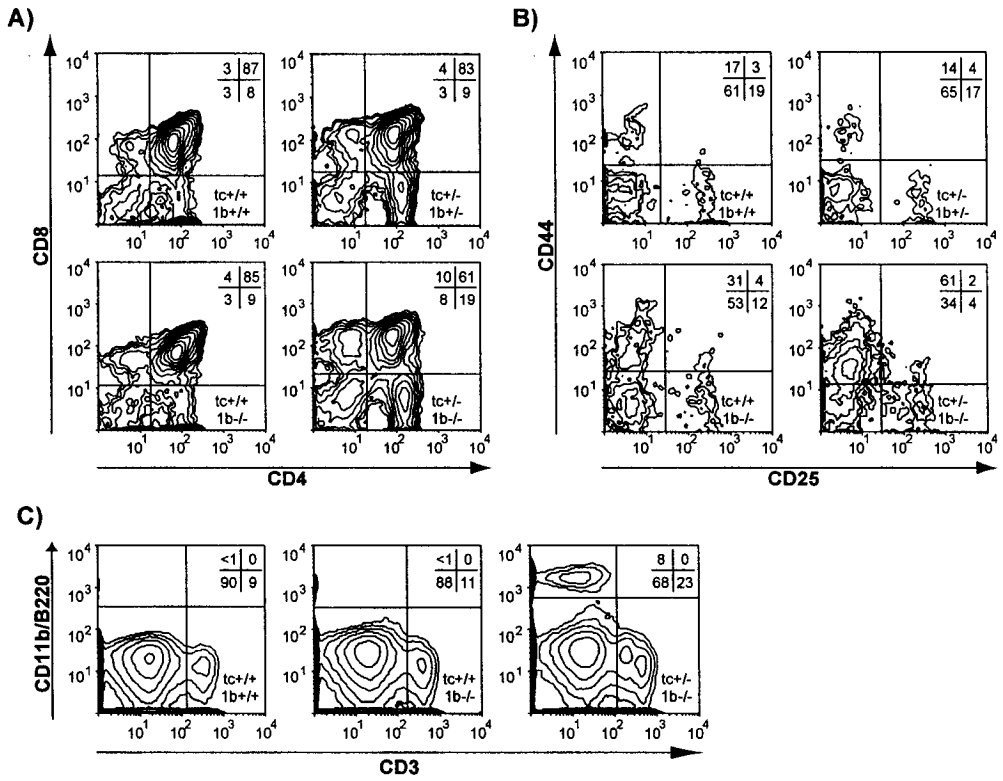


Figure 4.4 - Decrease in double-positive thymocytes and an infiltrate of CD11b+ cells in the tcptp+/-ptp1b-/- thymus.

A) Representative flow cytometry plots showing a relative increase in mature single positive thymocytes and a decrease in double positive thymocytes in tcptp+/-ptp1b-/- mice. n=5-12 per genotype. ***B)*** Representative flow cytometry plots showing a relative increase in the DN1 subpopulation in tcptp+/-ptp1b-/- thymus. n=5-12 per genotype. ***C)*** Representative flow cytometry plots showing an infiltration by CD11b+ cells in tcptp+/-ptp1b-/- thymus. n=3 for both tcptp+/+ptp1b+/+ and tcptp+/+ptp1b-/- mice; n=6 for tcptp+/-ptp1b-/-.

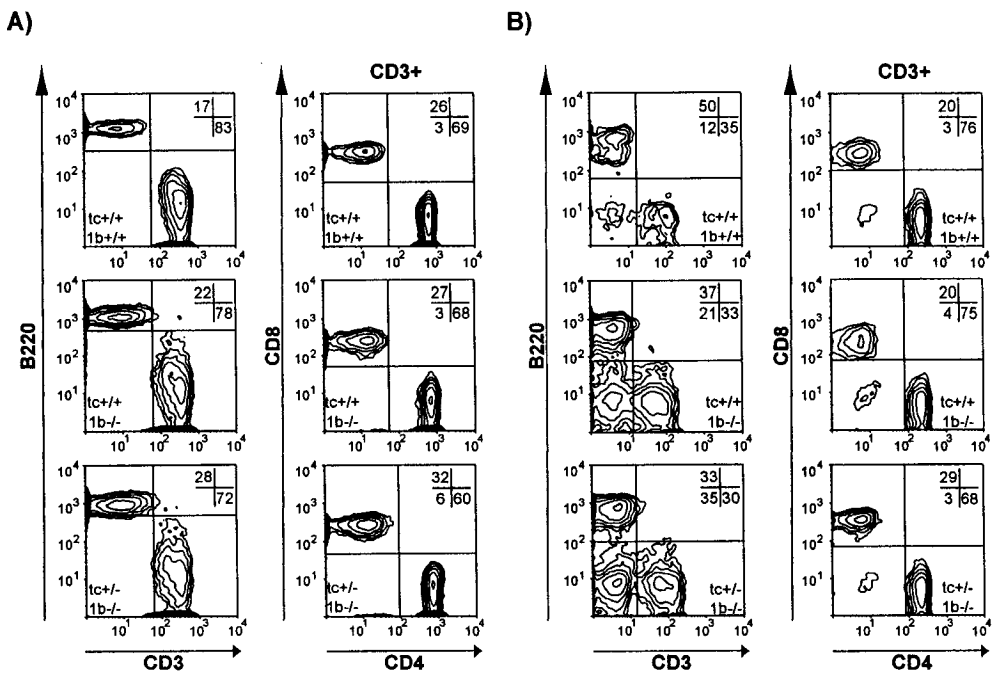


Figure 4.5 - Mild differences in the percentages of T lineage cells in axillary lymph nodes and spleen.

A) Representative flow cytometry plots from axillary lymph nodes showing an increase in the B to T cell ratio and a decrease in the CD4+ to CD8+ T cell ratio in tcptp+/-ptp1b-/- mice. n=9-12 per genotype. ***B)*** Representative flow cytometry plots from spleen showing decreases in both B to T cell and CD4+ to CD8+ T cell ratios in tcptp+/-ptp1b-/- mice. n=9-12 per genotype.

number of $B220^+$ cells rather than a loss of $CD3^+$ cells. When the $CD3^+$ cells were divided into $CD4^+$ and $CD8^+$, there was a reproducible decrease in the CD4/CD8 ratio, a phenomenon that is seen in inflammatory disease, such as graft-versus-host disease [387, 388]. However, we did not see a complete reversal of the ratio, indicating that the mechanism for the relative increase in $CD8^+$ cells is not likely to be the same.

Similar to axillary LN, the splenic *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ T cells also showed a decreased CD4/CD8 ratio (Fig. 4.5*b*). The percentage of $B220^+$ cells was decreased, likely reflecting the increase in myeloid cells as well as other unmarked cells (Fig. 4.3*b*). However, the absolute numbers of both $B220^+$ and $CD3^+$ populations were rather increased by at least two-fold due to the massive splenomegaly (table 4.2), indicating that either the *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ thymus is still able to maintain sufficient thymopoiesis, or there is extrathymic T cell development in the mouse, similar to the mice with T cells transgenic for OncostatinM [337], a cytokine that signals through Jak1 and Jak2.

4.4.5 Enhanced IFN-γ pathway.

Given that both TC-PTP and PTP-1B are known to regulate the IFN-γ signalling pathway at the level of Jak1, Stat1 and Jak2 [7, 128, 131], we wished to determine the IFN-γ responsiveness of *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ spleen-derived macrophages. Cells were incubated for 48 hours with 100U/ml IFN-γ, and nitrite production was measured in the supernatant and compared to unstimulated controls. Macrophages derived from *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ spleen produced on average three times more NO than *tcptp*$^{+/+}$ *ptp1b*$^{+/+}$ controls and approximately twice as much as either *tcptp*$^{+/-}$ *ptp1b*$^{+/-}$ or *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$ cells (Fig. 4.6*a*). Interestingly, cells derived from *tcptp*$^{-/-}$ *ptp1b*$^{+/+}$ mice did not produce more NO than *tcptp*$^{+/+}$ *ptp1b*$^{+/+}$ controls, indicating that the effect seen in *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ cells was more than additive. The initial report by Simoncic et al. [131] looked at signalling and iNOS upregulation in bone marrow-derived macrophages using 1000U/ml IFN-γ, which may explain the differences with the results shown here.

To see if the increased NO production stemmed from enhanced Stat1 phosphorylation, we stimulated spleen-derived macrophages with 100 U/ml IFN-γ for 10, 30 or 60 minutes and looked for Stat1 activation. As compared to *tcptp*$^{+/-}$ *ptp1b*$^{+/-}$ or *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$ cells, Stat1 phosphorylation was significantly enhanced in macrophages

Genotype (tc-ptp ptp-1b)	Total (10^6 cells)	$CD3^+$	$CD3^+$ $CD4^+$	$CD3^+$ $CD8^+$	$CD4^+$ $CD8^+$	Lin-	$B220^+$	$CD11b^+$
Thymus								
+/+ +/+	84.3	8.3	5.7	2.6	72.9	2.1	N.D.	0.19
	± 4.0	± 0.6	± 0.4	± 0.3	± 0.8	± 0.1		± 0.03
+/+ -/-	65.4	9.6	6.7	2.9	54.4	1.9	N.D.	0.35
	± 3.8	± 0.4	± 0.3	± 0.2	± 0.6	± 0.2		± 0.09
+/- -/-	14.7	4.8	3.3	1.6	9.0	1.2	N.D.	1.03
	± 2.2	± 0.4	± 0.3	± 0.2	± 0.5	± 0.2		± 0.20
LN								
+/+ +/+	4.0	3.0	2.1	0.9	N.D.	N.D.	0.6	N.D.
	± 0.1	± 0.1	± 0.2	± 0.1			± 0.1	
+/+ -/-	7.6	5.0	3.5	1.7	N.D.	N.D.	1.5	N.D.
	± 2.1	± 0.3	± 0.3	± 0.4			± 0.3	
+/- -/-	13.2	8.6	5.5	3.4	N.D.	N.D.	3.4	N.D.
	± 2.5	± 0.8	± 0.6	± 0.4			± 0.9	
Spleen								
+/+ +/+	103	31.1	21.9	9.8	N.D.	N.D.	42.3	
	± 10.6	± 5.6	± 4.7	± 1.2			± 2.9	
+/+ -/-	171	55.9	33.9	21.6	N.D.	N.D.	73.1	
	± 14.2	± 12.9	± 6.8	± 5.3			± 18.9	
+/- -/-	256	80.5	50.9	29.11	N.D.	N.D.	84.4	
	± 28.0	± 25.7	± 16.3	± 9.5			± 19.2	

Table 4.2 – Increase in mature cells in $tcptp^{+/-}$ $ptp1b^{-/-}$ mice.

Mean ± SE of the absolute numbers of different cells in thymus, lymph nodes and spleen of *$tcptp^{+/+}$ $ptp1b^{+/+}$*, *$tcptp^{+/+}$ $ptp1b^{-/-}$*, and *$tcptp^{+/-}$ $ptp1b^{-/-}$* mice.

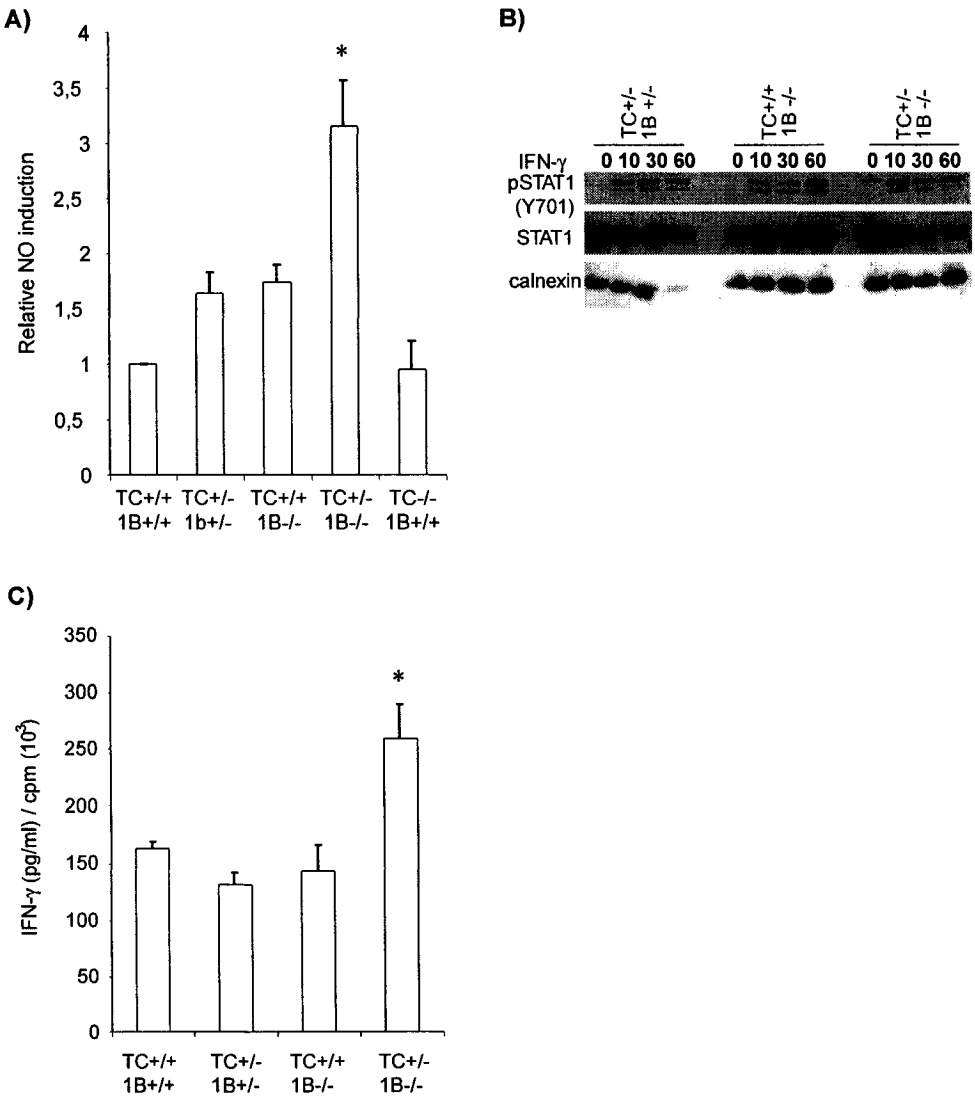


Figure 4.6 - Hyperactive IFN-γ pathway in tcptp+/- ptp1b-/- mice.

A) Nitrite production in response to 100 U/ml IFN-γ by spleen-derived macrophages. Bars represent mean ± SE from three independent experiments. n=3-5 per genotype. * $p<0.05$ tcptp+/- ptp1b-/- compared to other genotypes. ***B)*** Representative western blot showing increased Stat phosphorylation after stimulation with 100 U/ml IFN-γ in spleen-derived macrophages. Similar results were obtained in three separate experiments. ***C)*** IFN-γ production in supernatant by CD90.2+ spleen cells. The data are expressed as pg/ml IFN-γ per 1000 cpm in mitogen assay. Bars represent mean ± SE from two independent experiments. n=3-5 per genotype. * $p<0.02$ tcptp+/- ptp1b-/- compared to other genotypes.

derived from *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$ animals, especially at the earliest time point. At the later time points other mechanisms of negative control, for example, SOCS1 could attenuate the signal independent of TC-PTP and PTP-1B.

The *tcptp*$^{-/-}$ *ptp1b*$^{+/+}$ mice had increased levels of circulating IFN-γ shortly before death [301]. Also, their splenic T cells produced more IFN-γ on a per cell basis after mitogenic stimulation. We did not detect any serum IFN-γ in unstimulated *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ animals (data not shown). However, given the slower progression of the phenotype, it is still possible that IFN-γ could be detectable at later stage. Instead, we decided to determine the capacity of *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ T cells to produce IFN-γ in culture. Given that stimulation with Concanavalin A (ConA) or with anti-CD3/CD28 antibodies not only induces IFN-γ production but also proliferation, we decided to normalize the IFN-γ detected in supernatant to the cell proliferation as determined by ^{3}H-thymidine incorporation. IFN-γ production by control splenocytes (*tcptp*$^{+/+}$ *ptp1b*$^{+/+}$, *tcptp*$^{+/-}$ *ptp1b*$^{+/-}$, or *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$) was essentially equal, whereas *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ T cells produced almost twice as much IFN-γ when normalized to DNA synthesis. The total amount of IFN-γ in the supernatant was equivalent to the amount of IFN-γ in control supernatants; however, a smaller proportion of *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ cells proliferated as indicated by an approximately 70% decrease in ^{3}H-thymidine incorporation.

4.5 DISCUSSION

The results presented here demonstrate that TC-PTP and PTP-1B play a cooperative role during embryonic development as well as in macrophage development and activation after birth. The removal of one copy of TC-PTP on *ptp1b*$^{-/-}$ background revealed a gene dosage effect that had previously gone undetected in *tcptp*$^{+/-}$ mice. In these *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ mice, we were able to observe the collaborative effect of the two enzymes in macrophage development *in vivo* as well as in culture with CSF-1. Our data also demonstrate that IFN-γ signalling is potentiated in *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ macrophages as compared to either *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$ or *tcptp*$^{+/-}$ *ptp1b*$^{+/-}$ cells.

We have recently shown that macrophage development is affected in *ptp1b*$^{-/-}$ mice [382] (see Chapter 3). The loss of PTP-1B sensitized bone marrow precursors to CSF-1 as shown by an increased number of colonies in methylcellulose. The data presented here indicate that the effects of TC-PTP and PTP-1B on CSF-1 signalling are non-redundant, as *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ bone marrow presented even more colonies in response to CSF-1 than that of *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$ mice. These data are corroborated by findings from our laboratory indicating that TC-PTP dephosphorylated CSF-1R [383]. It therefore appears that both TC-PTP and PTP-1B negatively regulate CSF-1R signalling and thereby macrophage differentiation. This could occur at different levels: TC-PTP appears to control only dephosphorylation of the receptor whereas PTP-1B may also play a role in receptor recycling [382]. Such a role for PTP-1B in receptor maturation has been proposed in the context of Flt3, another member of the RTK Class III family [373]. The SH2-containing phosphatase-1 (SHP-1) is another cytoplasmic phosphatase that has been reported to control CSF-1 signalling [200] and is likely to exert some role also in the absence of TC-PTP and PTP-1B.

IFN-γ signalling is mediated by Jak1 and Jak2, two Janus family kinases [12]. A deletion of either one results in decreased IFN-γ responses [59-61]. Activation of Jak1 and Jak2 leads to the phosphorylation of the receptor as well as the downstream transcription activator Stat1, the presence of which is also required for proper IFN-γ signalling [77, 78]. Stat1 and Jak1 are reported substrates for TC-PTP [128, 131] while Jak2 has been shown to be dephosphorylated by PTP-1B [7]. The simultaneous ablation of TC-PTP and PTP-1B is therefore likely to greatly enhance and/or prolong IFN-γ

signalling. Indeed, we have shown here that *tcptp*$^{+/-}$ *ptp1b*$^{+/+}$ macrophages are more sensitive to IFN-γ than either *tcptp*$^{+/-}$ *ptp1b*$^{+/-}$ or *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$ controls. This increased sensitivity, coupled to the apparently increased capacity of T cells to produce IFN-γ, is likely an important mechanism explaining the decreased survival of *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ mice. The ablation of other negative regulators of IFN-γ signalling, SOCS1 and PIAS1, also results in decreased survival [158, 159, 177], although in the case of PIAS1, the effect was much milder, potentially due to the restricted effect on IFN-γ signalling (less than 10% IFN-γ inducible genes were actually affected by the deletion of PIAS1) [177]. In parallel, increasing IFN-γ leads to autoimmune inflammation as shown by tissue-specific transgenic expression in muscle [330], epidermis [331], pancreatic beta cells [332] and hepatocytes [333].

The cause of embryonic lethality in *tcptp*$^{-/-}$ *ptp1b*$^{-/-}$ mice is currently still under investigation. The timing of their death (~E10.5) would point to a defect in vasculogenesis or early definitive haematopoiesis [389-391]. However, none of the published substrates of either TC-PTP or PTP-1B is expected to have a direct effect on vasculogenesis, and the known hematopoietic defects would probably not result in embryonic death. It is possible that a completely new pathway is involved. Another interesting possibility is the interplay between IFN-γ and TGF-β. IFN-γ signalling results in decreased TGF-β synthesis and in decreased signalling downstream of TGF-β family receptors [389, 392, 393]. Ablation of either TGF-β, TGF-β receptor II, endoglin (another TGF-β receptor), or Smad5 (an important signal transducer downstream of TGF-β receptors) leads to embryonic death at E9.5-E10.5 due to defective vasculogenesis and cardiac development. The presumably enhanced IFN-γ signalling together with increased IFN-γ production in *tcptp*$^{-/-}$ *ptp1b*$^{-/-}$ embryos could decrease TGF-β signalling to a level which would be insufficient for normal embryonic development.

Another potential pathway is the integrin signalling. Deficiency in α_4 [394] or β_1 [395] integrins leads to embryonic death at implantation or E12-13, respectively. PTP-1B is known to regulate integrin signalling through dephosphorylation of Src [249, 396]. On the other hand, $\alpha_1\beta_1$ integrins activate TC-PTP [230]. The physiological consequences of either phosphatase in the context of integrin signalling are still unclear: fibroblasts with no functional PTP-1B [249] or treated with PTP-1B inhibitors [396] show deficiencies in

spreading and migration on fibronectin, whereas activation of TC-PTP decreased EGF-mediated proliferation and increased anchorage dependence of transformed cells [230]. Simultaneous deletion of both in *tcptp*$^{-/-}$ *ptp1b*$^{-/-}$ embryos could result in defective cell migration and loss of proper growth control.

Lastly, interferons are currently used for treatment of chronic myelogenous leukemia (CML) [397], adult T cell leukemia [398], Kaposi's sarcoma [399], and hairy cell leukemia [400] as well as hepatocellular carcinoma [401] and melanoma [402]. In the case of CML, a negative correlation has been shown between responsiveness to IFN-α and TC-PTP activity [381, 403]. Therefore, inhibiting TC-PTP, and simultaneously PTP-1B, could increase the sensitivity of malignant cells to interferons.

In conclusion, we have demonstrated here that TC-PTP and PTP-1B have a cooperative and non-redundant role in IFN-γ and CSF-1R signalling. This became evident as an increased responsiveness of *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ macrophages to IFN-γ and increased monocytic colony formation in response to CSF-1 by *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ bone marrow cells. Our results reveal the previously undetected haploinsufficiency of TC-PTP and suggest that simultaneously inhibiting TC-PTP and PTP-1B could have beneficial effects as adjuvant to IFN treatment.

4.6 ACKNOWLEDGEMENTS

We thank Rosemary Siegrist-Johnstone and Ailsa Lee Loy for their excellent technical assistance, as well as Eric Massicotte and Martine Dupuis from Institut des recherches cliniques de Montréal (IRCM) and Ken McDonald for their valuable help with flow cytometry.

5 CHAPTER V – DISCUSSION

The aim of this thesis was to study the role of two structurally similar phosphatases, T cell protein tyrosine phosphatase (TC-PTP; also known as PTPN2) and protein tyrosine phosphatase 1B (PTP-1B; also known as PTPN1), in the regulation of monocyte/macrophage development and activation. Both enzymes are strongly expressed in the hematopoietic system; TC-PTP has been previously established to influence T and B lymphopoiesis [2] as well as IFN-γ signalling in bone marrow derived macrophages [131], whereas until recently, PTP-1B has had no known role in the immune system beyond that of regulating interferon signalling in fibroblasts [7]. Chapter 2 of the thesis defines how the loss of TC-PTP in mice results in dysregulated response to LPS and accumulation of activated inflammatory cells as well as end-products of IFN-γ signalling. Chapter 3 describes a new role for PTP-1B in monocyte/macrophage development and activation as a regulator of CSF-1R signalling. Finally, in Chapter 4, we have shown that TC-PTP and PTP-1B play complementary roles in macrophage development and activation at the level of CSF-1R and IFN-γ signalling, resulting in the death of *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ mice.

5.1 TC-PTP AND PTP-1B IN HEMATOPOIESIS

Although tyrosine phosphorylation is common only in multicellular organisms [113], its control is crucial for the proper functioning of several systems, including embryogenesis, metabolism, cell growth and differentiation, and depends on the reciprocal activity of tyrosine kinases and phosphatases. In the immune system, the main focus has probably been the regulation of T and B cell receptor signaling, and several phosphatases have been implicated in the dephosphorylation of one or another component of the pathway [118, 183, 404, 405]. However, cytokine signaling is also an important part of hematopoiesis and immune homeostasis. SOCS and PIAS are known protein regulators of cytokine signaling; nevertheless, protein tyrosine phosphatases also play an important role through dephosphorylation of the signaling components, including the receptor chains, Jak kinases, and Stats. PTP-1B and TC-PTP have been previously shown to

control cytokine signaling [5-7, 125, 128, 131]. Their effects on hematopoiesis and immune function have not been thoroughly defined, though.

Conditional or hematopoeitic lineage-specific knockout mice for TC-PTP or PTP-1B have yet to be generated, and our current understanding of the function of TC-PTP and PTP-1B in immune development is based on the studies of the *tcptp*$^{-/-}$ and *ptp1b*$^{-/-}$ mice. The latter are viable, but present moderate splenomegaly and lymphadenopathy (approximately 2-fold increase in cell number in both cases; see chapter 3 and refs. [327, 382]). Conversely, *tcptp*$^{-/-}$ mice die within 3-5 weeks of birth and display more striking symptoms, including runting, severe splenomegaly, thymic atrophy, diarrhea and hunched posture [2]. Nevertheless, both strains of mice have several abnormalities in the hematopoietic compartment, some of which are similar, others opposite. The different lineages and how they are affected by TC-PTP and PTP-1B are depicted in Fig. 5.1.

The initial paper describing the *tcptp*$^{-/-}$ mouse phenotype reported an increased percentage of F4-80^{+} cells in the spleen [2]. Work presented in the Chapter 2 of this thesis described mononuclear infiltrates to several organs, as well as the presence of iNOS mRNA and protein in some of these organs, strongly suggesting that these mononuclear cells comprised monocyte/macrophage-lineage cells (see Figs. 2.1, 2.2 and 2.6). Furthermore, we showed that *tcptp*$^{-/-}$ macrophages, whether taken directly from the spleen or derived *in vitro*, were highly sensitive to LPS, indicative of an activated phenotype (see Fig. 2.8). Our data are further corroborated by other data from our laboratory, suggesting that TC-PTP is a negative regulator of CSF-1 signalling [383]. Together, these results clearly demonstrate that TC-PTP plays an important role in monocyte/macrophage development.

Given that Jak2 is involved in signalling downstream of several myeloid cytokines (IL-3, G-CSF and GM-CSF) [61, 364, 365], we expected that PTP-1B as a regulator of Jak2 phosphorylation would also influence myeloid development. Indeed, as described in Chapter 3 of this thesis, mice deficient in PTP-1B have increased numbers of monocyte/macrophage lineage cells and the precursor cells in *ptp1b*$^{-/-}$ bone marrow preferentially develop into monocytic rather than granulocytic colonies when in presence of multi-lineage growth factors (see Figs. 3.1*a* and 3.3). Somewhat surprisingly, however,

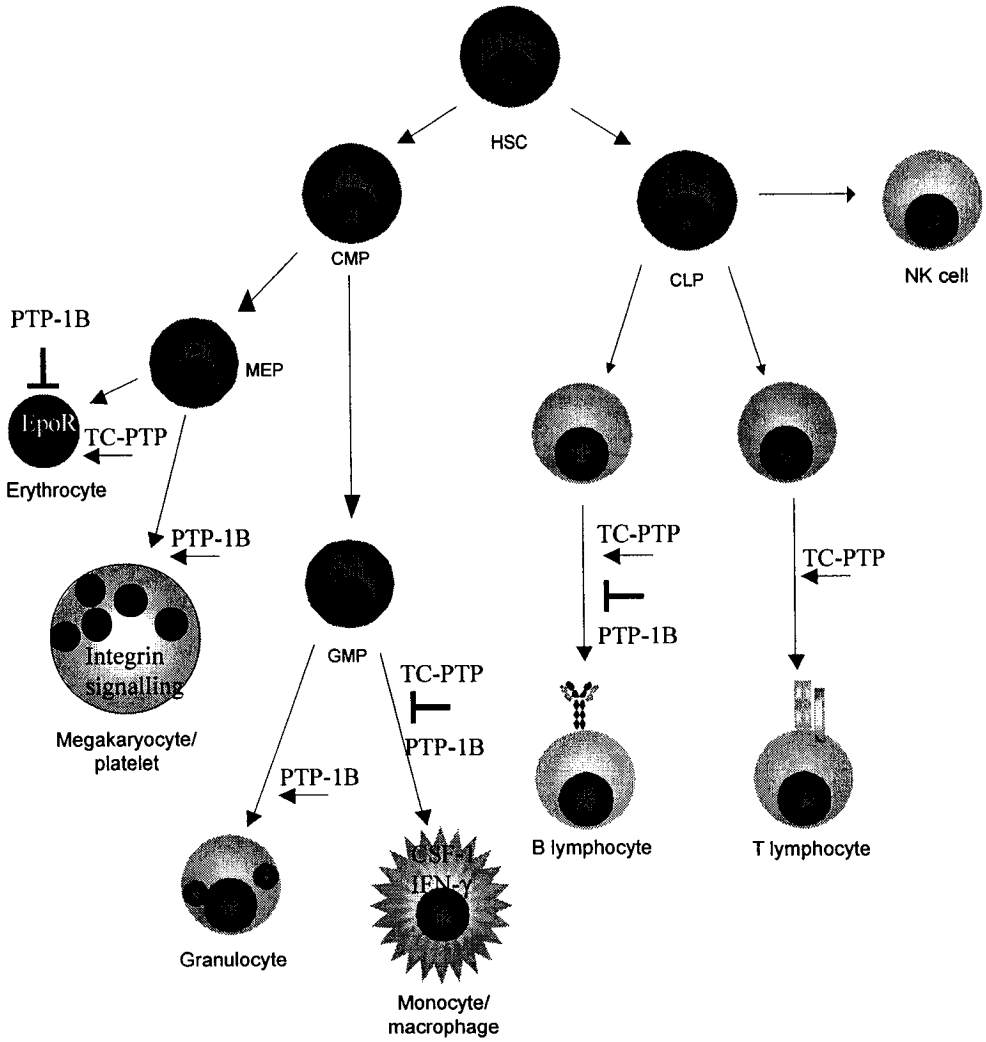


Figure 5.1 - TC-PTP and PTP-1B in hematopoiesis.

A schematic of the different hematopoietic lineages with the roles of TC-PTP and PTP-1B indicated as arrows (positive role) or T-bars (negative role), including the phosphatase substrate involved, if known.

the major cytokine responsible for this phenotype appeared to be CSF-1 as we detected increased colony formation in response to CSF-1 but not to G-CSF or GM-CSF (see Fig. 3.2*a*) as well as increased proliferation (see Fig. 3.2*b*). Moreover, the catalytic site tyrosine of the CSF-1R was hyperphosphorylated in *ptp1b*$^{-/-}$ bone marrow derived macrophages in response to CSF-1, and similar results were also observed in macrophage cell lines overexpressing the catalytic mutant PTP-1B DA (see Fig. 3.2*c*). Thus, our results indicate that PTP-1B is a specific regulator of monocyte/macrophage development through its ability to modulate CSF-1R phosphorylation.

The consequences of such an effect of PTP-1B on macrophage development remain unclear. The *ptp1b*$^{-/-}$ mice are viable and present no visible inflammatory phenotype when kept in a specific pathogen free facility. This is in striking contrast to *tcptp*$^{-/-}$ mice [2] or SHP-1-deficient *me/me* mice [194, 195, 200], both of which have greatly shortened life-spans and appear to play a role in macrophage development through CSF-1 [200, 383]. Moreover, when stimulated with LPS, *ptp1b*$^{-/-}$ macrophages displayed enhanced sensitivity as attested by increased NO production (Figs. 3.4*a* and *b*), and *ptp1b*$^{-/-}$ mice were accordingly more susceptible to LPS-induced cytokine production and septic shock (Figs. 3.4*e* and *f*).

One possible difference between the *tcptp*$^{-/-}$ and *ptp1b*$^{-/-}$ macrophages in this respect could reside in the accessibility to stimulus (see Fig. 5.2. for model). First, *ptp1b*$^{-/-}$ mice have no obvious defects in T cell development (Chapter 4 and ref. [327]). Therefore, although IFN-γ signalling is enhanced (Fig. 3.4*c*) in *ptp1b*$^{-/-}$ macrophages, there would be no constant source of IFN-γ, which would presumably still be necessary for macrophage activation. In contrast, *tcptp*$^{-/-}$ mice display thymic atrophy, and their T cells produce high amounts of IFN-γ upon activation (Fig. 2.5). Moreover, even in the absence of external stimulus, IFN-γ mRNA could be detected in several organs in *tcptp*$^{-/-}$ mice (Figs. 2.1, 2.3), and significant amounts of IFN-γ protein were detectable in circulation (Fig. 2.7). Furthermore, it is possible that some of the IFN-γ was produced by non-T cells in *tcptp*$^{-/-}$ mice (A. Bourdeau, personal communication), which would induce inflammation in the producing organ, similar to, for example, the transgenic animals expressing IFN-γ in pancreatic β-cells [332].

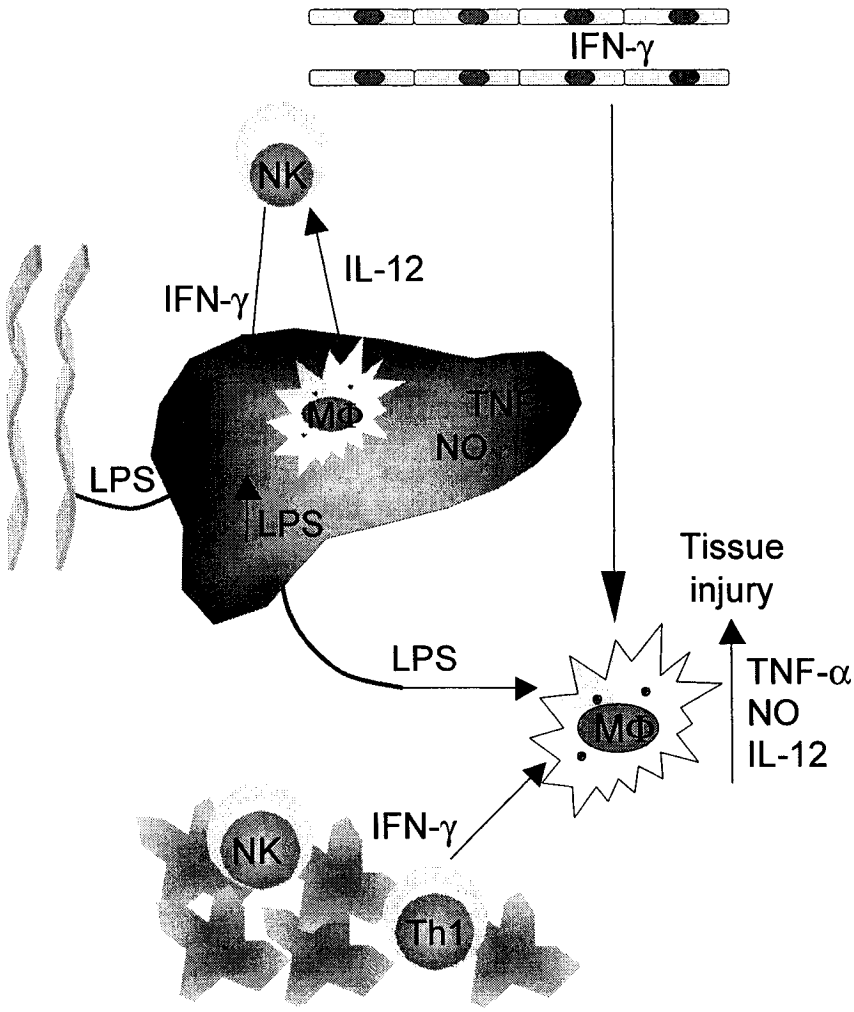


Figure 5.2 - Macrophage activation in vivo in tcptp-/- animals.

Due to the thinning of the intestinal epithelium, LPS leaks into the portal circulation and accumulates in the liver. Hepatic macrophages (Kupffer cells) come in contact with LPS and produce IL-12, which will in turn stimulate IFN production by, e.g., liver NK cells. This will further activate the Kupffer cells to produce cytotoxic agents, including NO and TNF-α. As the levels of LPS increase in the liver, some will leak over into systemic circulation and activate macrophages in other tissues. These have already been primed by IFN-γ produced either by inflammatory Th1 or NK cells or potentially epithelial or stromal cells. In the absence of negative regulation, the cycle will go on and result in the accumulation of inflammatory mediators.

Second, TC-PTP is involved in cell cycle regulation, and its absence is known to delay cell cycle progression [3]. In the case of a high turnover population, such as gut epithelium, the slower proliferation rate could result in decreased cellularity and, consequently, in increased permeability of the epithelial layer, which would allow for increased transit of LPS to the portal system. Such leakiness of the gut lining and accumulation of LPS in the liver is seen in GVHD [286, 345, 355, 356], and increased gut permeability has also been proposed as a mechanism in inflammatory bowel disease [406]. In *ptp1b*$^{-/-}$ mice, there is no apparent change to the intestines (K.M.H. unpublished observations) and *ptp1b*$^{-/-}$ macrophages would therefore not receive significant amounts of stimulus unless the mice are injected with LPS.

In addition to the data presented above, other publications have linked TC-PTP and PTP-1B to the development of other hematopoietic lineages. B cell development is influenced by both TC-PTP and PTP-1B but in opposite ways: *tcptp*$^{-/-}$ bone marrow becomes depleted of B cell precursors with age [2], while that of *ptp1b*$^{-/-}$ mice presents an increased number of B220^{+} cells, particularly immature IgM^{-} B cells [327], which makes them more susceptible to develop B cell lymphomas on *p53*$^{-/-}$ background. Erythropoiesis is likely to be affected by both TC-PTP and PTP-1B as well: *tcptp*$^{-/-}$ mice are anemic [2], possibly secondary to IFN-γ [407], while PTP-1B has been shown to dephosphorylate the Epo receptor [127]. In addition, PTP-1B is necessary during platelet activation [408]. Together, TC-PTP and PTP-1B influence the function and development of the majority of hematopoietic lineages.

5.2 TC-PTP AND PTP-1B – DIFFERENCES AND REDUNDANCIES

TC-PTP and PTP-1B have highly similar structures and catalytic activities, and although they are not found on the same chromosome in either man or mouse, their sequence similarities suggest a gene duplication event [116]. It is tempting to propose that such a duplication would enable tissue-specific control, and that the two enzymes would have very little overlapping function. However, both TC-PTP and PTP-1B are ubiquitously expressed, and can be found at high levels in hematopoietic as well as non-hematopoietic tissues [222, 324, 325]. Very little is currently known about the spatial and temporal

control of TC-PTP or PTP-1B expression. Induction of TC-PTP is associated with cell cycle progression [3, 253, 362] which explains why high levels of TC-PTP mRNA are detected in organs such as thymus and testes; however, it does not account for the differences seen in kidney (high) versus heart (low) versus liver (moderate), for example [222]. PTP-1B expression is suppressed by Egr1 but upregulated by Sp-family proteins [379] and Y box-binding protein (YB)-1 [409]. Moreover, increased PTP-1B expression has been detected in animal models for type II diabetes, Erb2-overexpressing breast cancer and CML [252, 410-413]. Both TC-PTP and PTP-1B can be phosphorylated [241, 414, 415], and PTP-1B activity can be further modulated by proteolytic cleavage [239].

Given the widespread effects of TC-PTP and PTP-1B in immune development, it is perhaps not surprising that the simultaneous deletion of both enzymes should be lethal during embryogenesis. Moreover, based on the *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ mice, at least macrophage development (Fig. 4.3) and IFN-γ signaling (Fig. 4.6) are regulated by the two enzymes in an additive manner. Activation of decidual macrophages is associated with embryo loss [416], and although such a model would not explain selective resorption of *tcptp*$^{-/-}$ *ptp1b*$^{-/-}$ embryos only, enhanced macrophage activation and IFN-γ signaling in the embryo could provide activation signals to the decidual macrophages. In addition, as discussed in Chapter 4, the enhanced IFN-γ signaling could prevent necessary TGF-β signals and thereby result in defective vasculogenesis and embryonic death. Both avenues are currently under study.

There are two possible explanations for the additive CSF-1 response seen in *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ bone marrow as compared to *ptp1b*$^{-/-}$ cells. One possibility is that both enzymes simply dephosphorylate the active site tyrosine, and the less phosphatase present, the higher the phosphorylation level of the site. However, this implies that quite large amounts of phosphatase activity are necessary for normal regulation. On the other hand, PTP-1B also appears to regulate receptor kinase maturation [373] through the regulation of the phosphorylation status. A recent report also indicates that PTP-1B could regulate receptor internalization through caveolin [417]. We therefore propose that the simultaneous removal of TC-PTP and PTP-1B would result in both enhanced and prolonged phosphorylation of the receptor, partly because of the removal of specific phosphatase activity, partly due to the modified receptor recycling.

IFN-γ signaling, on the other hand, would be enhanced in *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ macrophages due to the loss of specific phosphatase activity at several levels in the downstream signaling pathway: TC-PTP is known to dephosphorylate Jak1 and Stat1 [128, 131], whereas PTP-1B dephosphorylates Jak2 [7]. Thus, there is no common substrate in the IFN-γ signaling pathway, but the two enzymes rather cooperatively regulate several signaling components, so that the lack of both enzymes at the same time results in much stronger hyperphosphorylation of the last component (Stat1) and activation of the pathway (as seen by NO production) than the deletion of either one alone.

One of the major differences between *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ and *tcptp*$^{+/+}$ *ptp1b*$^{-/-}$ mice is the enhanced IFN-γ sensitivity and potential for IFN-γ production (Fig. 4.6). Both strains have increased numbers of primed macrophages, but only in the former do they result in fatal inflammation (Fig. 4.2). As discussed above (and presented in Fig. 5.2), perhaps the most simplified explanation lies in the accessibility to activating stimulus. Even moderate IFN-γ production or normally innocuous amounts of LPS in the presence of highly sensitized macrophages would result in uncontrolled activation, similar to what we have reported in *tcptp*$^{-/-}$ mice (Chapter 2). With respect to other hematopoietic lineages that might be affected in *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ mice, we did not detect any significant differences in the number of burst-forming units of erythroblasts (BFU-E), but the percentage of Ter119^{+} labeled cells was significantly lower, suggesting that the mice may have been anemic (unpublished observations). In accordance with the fact that the B lymphocyte phenotype of *tcptp*$^{-/-}$ and *ptp1b*$^{-/-}$ mice are essentially opposite, the relative B220^{+} populations were not significantly altered in the periphery (Fig. 4.5) and the bone marrow presented essentially normal numbers of B220^{+} cells (unpublished observations).

PTP-1B is best known as a metabolic regulator due to its ability to affect insulin [4, 244, 245], leptin [5, 125] and growth hormone [6] signaling. Recently, TC-PTP has also been linked to insulin signaling in fibroblasts [233, 234]; however, physiological readout for any cooperative role in metabolic regulation is still lacking. Metabolic studies in either *tcptp*$^{-/-}$ or *tcptp*$^{+/-}$ *ptp1b*$^{-/-}$ mice are complicated by the rather overwhelming immune phenotype, and the *tcptp*$^{+/-}$ *ptp1b*$^{+/-}$ mice might be best suited for more physiological analysis of the TC-PTP/PTP-1B interplay. Although *tcptp*$^{+/-}$ *ptp1b*$^{+/-}$ mice tend to be

smaller that their *tcptp*$^{+/+}$ *ptp1b*$^{+/+}$ littermates (unpublished observations), preliminary results using *tcptp*$^{+/-}$ mice do not suggest any significant differences in insulin sensitivity (M.L. Tremblay, personal communication). Further studies will be necessary to ascertain the extent to which TC-PTP contributes to the regulation of insulin signaling *in vivo*.

5.3 PTP-1B AND TC-PTP INHIBITORS

Due to the high structural homology in the catalytic domains of TC-PTP and PTP-1B, the small molecule inhibitors currently designed against PTP-1B have only moderate selectivity against TC-PTP. Therefore, when proposing uses for such inhibitors, it must be considered that both enzymes will be inhibited to a certain degree. This simultaneous inhibition can be a disadvantage but also an advantage, depending on the pathway that needs to be targeted.

Based on the work presented in Chapter 4, TC-PTP and PTP-1B cooperate in the regulation of IFN-γ signalling. Interferons are currently used for treatment of various malignancies, including chronic myelogenous leukemia (CML) [397], adult T cell leukemia [398], and hairy cell leukemia [400]. Of particular interest, a negative correlation has been shown between responsiveness of CML lines to IFN-α and TC-PTP activity [381, 403]. Moreover, PTP-1B expression is increased in CML due to Bcr-Abl signalling, although the consequences of the overexpression are unknown [252, 326]. Genetic ablation of IRF8, an IFN-γ responsive protein, in mice is associated with a CML-like syndrome [323]. It is therefore possible that as the overexpression of PTP-1B renders myeloid cells more resistant to IFN-γ, it also makes them more susceptible to adopt a CML phenotype, making overexpression of PTP-1B a pro-oncogenic event. Thus, simultaneous inhibition of TC-PTP and PTP-1B, could increase not only the sensitivity of CML cells to interferon treatment but also their differentiation through a CSF-1R independent mechanism. Similarly, PTP-1B/TC-PTP inhibitors could be used as adjuvant to interferon treatment in other malignancies.

On another note, SHP-1 activity has been shown to be regulated by *Leishmania donovani* [205, 206, 208] to decrease IFN-γ sensitivity of infected cells and help the parasite to escape detection. Inhibiting PTP-1B and TC-PTP activity in infected

macrophages should increase their activation and IFN-γ responsiveness and help in clearing the infection.

5.4 SUMMARY AND FUTURE DIRECTIONS

Our understanding of phosphatase function in general and the roles of TC-PTP and PTP-1B in particular has greatly changed over the past four and a half years that I have worked in Dr. Tremblay's laboratory. Novel data involve for example the roles of TC-PTP and PTP-1B in cytokine signalling, evidence for hematopoietic function of PTP-1B, regulation of phosphatase function by oxidization. The contribution of my research includes understanding the role of TC-PTP as a regulator of systemic inflammation (Chapter 2); defining PTP-1B as an important switch in myeloid development and modulation of CSF-1 signalling (Chapter 3); and first *in vivo* data on complementarities in TC-PTP and PTP-1B function (Chapter 4).

Clearly, several things remain to be elucidated. How do essentially nuclear (TC-PTP) and ER-localized (PTP-1B) enzymes access their plasma membrane-bound substrates? What is the true contribution of TC-PTP to metabolic signalling? Our mice are kept in specific pathogen free environment. What would be the consequences of subjecting *tcptp*$^{+/-}$ or *ptp1b*$^{-/-}$ mice to pathogens? Allergens? Since *ptp1b*$^{-/-}$ macrophages are activated, but express lower amounts of CD11b (a CR3 chain), would they be efficient at phagocytosis? Could they influence the clearance of intracellular pathogens? Are the mice immunocompromised despite (or perhaps due to) the increased macrophage activation?

In summary, then, the results of my doctoral research presented in this thesis demonstrate that TC-PTP and PTP-1B play independent and complementary roles in macrophage development and activation as well as the control of inflammatory responses and provide novel therapeutic approaches to myeloproliferative disorders and situations where stimulation of interferon signalling may be beneficial.

6 REFERENCES

1. Bourdeau, A., et al. Cytoplasmic protein tyrosine phosphatases, regulation and function: the roles of PTP1B and TC-PTP. Curr Opin Cell Biol. 2005. 17: 203-9.
2. You-Ten, K.E., et al. Impaired bone marrow microenvironment and immune function in T cell protein tyrosine phosphatase-deficient mice. J Exp Med. 1997. 186: 683-93.
3. Ibarra-Sanchez, M.J., et al. Murine embryonic fibroblasts lacking TC-PTP display delayed G1 phase through defective NF-kappaB activation. Oncogene. 2001. 20: 4728-39.
4. Elchebly, M., et al. Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. Science. 1999. 283: 1544-8.
5. Cheng, A., et al. Attenuation of leptin action and regulation of obesity by protein tyrosine phosphatase 1B. Dev Cell. 2002. 2: 497-503.
6. Gu, F., et al. Protein tyrosine phosphatase 1B attenuates growth hormone-mediated JAK2-STAT signaling. Mol Cell Biol. 2003. 23: 3753-62.
7. Myers, M.P., et al. TYK2 and JAK2 are substrates of protein-tyrosine phosphatase 1B. J Biol Chem. 2001. 276: 47771-4.
8. Jia, Z., et al. Structure of protein tyrosine phosphatase 1B in complex with inhibitors bearing two phosphotyrosine mimetics. J Med Chem. 2001. 44: 4584-94.
9. Chaiken, I.M. and W.V. Williams. Identifying structure-function relationships in four-helix bundle cytokines: towards de novo mimetics design. Trends Biotechnol. 1996. 14: 369-75.
10. Noguchi, M., et al. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. Cell. 1993. 73: 147-57.
11. Leonard, W.J. Cytokines and immunodeficiency diseases. Nat Rev Immunol. 2001. 1: 200-8.
12. Renauld, J.C. Class II cytokine receptors and their ligands: key antiviral and inflammatory modulators. Nat Rev Immunol. 2003. 3: 667-76.
13. Pestka, S., et al. The interferon gamma (IFN-gamma) receptor: a paradigm for the multichain cytokine receptor. Cytokine Growth Factor Rev. 1997. 8: 189-206.
14. Reilly, J.T. Class III receptor tyrosine kinases: role in leukaemogenesis. Br J Haematol. 2002. 116: 744-57.
15. Pixley, F.J. and E.R. Stanley. CSF-1 regulation of the wandering macrophage: complexity in action. Trends Cell Biol. 2004. 14: 628-38.
16. Liew, F.Y., et al. Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol. 2005. 5: 446-58.
17. Takeda, K. and S. Akira. Toll-like receptors in innate immunity. Int Immunol. 2005. 17: 1-14.
18. Schnare, M., et al. Toll-like receptors control activation of adaptive immune responses. Nat Immunol. 2001. 2: 947-50.

19. Suzuki, N., et al. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. Nature. 2002. 416: 750-6.
20. Suzuki, N., et al. IL-1R-associated kinase 4 is required for lipopolysaccharide-induced activation of APC. J Immunol. 2003. 171: 6065-71.
21. Hacker, H., et al. Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF)6. J Exp Med. 2000. 192: 595-600.
22. Horng, T., et al. The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. Nature. 2002. 420: 329-33.
23. Yamamoto, M., et al. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature. 2002. 420: 324-9.
24. Poltorak, A., et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998. 282: 2085-8.
25. Nagai, Y., et al. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. Nat Immunol. 2002. 3: 667-72.
26. Barton, G.M., et al. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nat Immunol. 2006. 7: 49-56.
27. Leadbetter, E.A., et al. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. Nature. 2002. 416: 603-7.
28. Ohashi, K., et al. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. J Immunol. 2000. 164: 558-61.
29. Dybdahl, B., et al. Inflammatory response after open heart surgery: release of heat-shock protein 70 and signaling through toll-like receptor-4. Circulation. 2002. 105: 685-90.
30. Vabulas, R.M., et al. HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. J Biol Chem. 2002. 277: 15107-12.
31. Sims, J.E. IL-1 and IL-18 receptors, and their extended family. Curr Opin Immunol. 2002. 14: 117-22.
32. Schmitz, J., et al. IL-33, an Interleukin-1-like Cytokine that Signals via the IL-1 Receptor-Related Protein ST2 and Induces T Helper Type 2-Associated Cytokines. Immunity. 2005. 23: 479-90.
33. Wald, D., et al. SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling. Nat Immunol. 2003. 4: 920-7.
34. Gadina, M., et al. Signaling by type I and II cytokine receptors: ten years after. Curr Opin Immunol. 2001. 13: 363-73.
35. O'Shea, J.J., et al. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. Cell. 2002. 109 Suppl: S121-31.
36. Hilkens, C.M., et al. A region encompassing the FERM domain of Jak1 is necessary for binding to the cytokine receptor gp130. FEBS Lett. 2001. 505: 87-91.
37. Zhou, Y.J., et al. Unexpected effects of FERM domain mutations on catalytic activity of Jak3: structural implication for Janus kinases. Mol Cell. 2001. 8: 959-69.
38. Chen, M., et al. Complex effects of naturally occurring mutations in the JAK3 pseudokinase domain: evidence for interactions between the kinase and pseudokinase domains. Mol Cell Biol. 2000. 20: 947-56.

39. Saharinen, P., et al. Regulation of the Jak2 tyrosine kinase by its pseudokinase domain. Mol Cell Biol. 2000. 20: 3387-95.
40. Yeh, T.C., et al. A dual role for the kinase-like domain of the tyrosine kinase Tyk2 in interferon-alpha signaling. Proc Natl Acad Sci U S A. 2000. 97: 8991-6.
41. Takeuchi, O., et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol. 2002. 169: 10-4.
42. Alexopoulou, L., et al. Hyporesponsiveness to vaccination with Borrelia burgdorferi OspA in humans and in TLR1- and TLR2-deficient mice. Nat Med. 2002. 8: 878-84.
43. Takeuchi, O., et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity. 1999. 11: 443-51.
44. Schwandner, R., et al. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J Biol Chem. 1999. 274: 17406-9.
45. Underhill, D.M., et al. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature. 1999. 401: 811-5.
46. Means, T.K., et al. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. J Immunol. 1999. 163: 6748-55.
47. Schulz, O., et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature. 2005. 433: 887-92.
48. Alexopoulou, L., et al. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 2001. 413: 732-8.
49. Hayashi, F., et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature. 2001. 410: 1099-103.
50. Takeuchi, O., et al. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int Immunol. 2001. 13: 933-40.
51. Heil, F., et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science. 2004. 303: 1526-9.
52. Hemmi, H., et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol. 2002. 3: 196-200.
53. Hemmi, H., et al. A Toll-like receptor recognizes bacterial DNA. Nature. 2000. 408: 740-5.
54. Hemmi, H., et al. The roles of Toll-like receptor 9, MyD88, and DNA-dependent protein kinase catalytic subunit in the effects of two distinct CpG DNAs on dendritic cell subsets. J Immunol. 2003. 170: 3059-64.
55. Krug, A., et al. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. Immunity. 2004. 21: 107-19.
56. Hasan, U., et al. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. J Immunol. 2005. 174: 2942-50.
57. Yarovinsky, F., et al. TLR11 activation of dendritic cells by a protozoan profilin-like protein. Science. 2005. 308: 1626-9.
58. Zhang, D., et al. A toll-like receptor that prevents infection by uropathogenic bacteria. Science. 2004. 303: 1522-6.

59. Rodig, S.J., et al. Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. Cell. 1998. 93: 373-83.
60. Neubauer, H., et al. Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. Cell. 1998. 93: 397-409.
61. Parganas, E., et al. Jak2 is essential for signaling through a variety of cytokine receptors. Cell. 1998. 93: 385-95.
62. Nosaka, T., et al. Defective lymphoid development in mice lacking Jak3. Science. 1995. 270: 800-2.
63. Park, S.Y., et al. Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. Immunity. 1995. 3: 771-82.
64. Shimoda, K., et al. Tyk2 plays a restricted role in IFN alpha signaling, although it is required for IL-12-mediated T cell function. Immunity. 2000. 13: 561-71.
65. Karaghiosoff, M., et al. Partial impairment of cytokine responses in Tyk2-deficient mice. Immunity. 2000. 13: 549-60.
66. Lacronique, V., et al. A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. Science. 1997. 278: 1309-12.
67. Peeters, P., et al. Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9;12) in a lymphoid and t(9;15;12) in a myeloid leukemia. Blood. 1997. 90: 2535-40.
68. Schwaller, J., et al. Transformation of hematopoietic cell lines to growth-factor independence and induction of a fatal myelo- and lymphoproliferative disease in mice by retrovirally transduced TEL/JAK2 fusion genes. Embo J. 1998. 17: 5321-33.
69. Ho, J.M., et al. Fusion of the ets transcription factor TEL to Jak2 results in constitutive Jak-Stat signaling. Blood. 1999. 93: 4354-64.
70. Jones, A.V., et al. Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. Blood. 2005. 106: 2162-8.
71. Kralovics, R., et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med. 2005. 352: 1779-90.
72. Wernig, G., et al. Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. Blood. 2006.
73. Levine, R.L., et al. The JAK2V617F activating mutation occurs in chronic myelomonocytic leukemia and acute myeloid leukemia, but not in acute lymphoblastic leukemia or chronic lymphocytic leukemia. Blood. 2005. 106: 3377-9.
74. Witthuhn, B.A., et al. Involvement of the Jak-3 Janus kinase in signalling by interleukins 2 and 4 in lymphoid and myeloid cells. Nature. 1994. 370: 153-7.
75. Johnston, J.A., et al. Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2. Nature. 1994. 370: 151-3.
76. Kawamura, M., et al. Molecular cloning of L-JAK, a Janus family protein-tyrosine kinase expressed in natural killer cells and activated leukocytes. Proc Natl Acad Sci U S A. 1994. 91: 6374-8.
77. Durbin, J.E., et al. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell. 1996. 84: 443-50.

78. Meraz, M.A., et al. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell. 1996. 84: 431-42.
79. Park, C., et al. Immune response in Stat2 knockout mice. Immunity. 2000. 13: 795-804.
80. Takeda, K., et al. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. Proc Natl Acad Sci U S A. 1997. 94: 3801-4.
81. Alonzi, T., et al. Essential role of STAT3 in the control of the acute-phase response as revealed by inducible gene inactivation [correction of activation] in the liver. Mol Cell Biol. 2001. 21: 1621-32.
82. Takeda, K., et al. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. Immunity. 1999. 10: 39-49.
83. Sano, S., et al. Stat3 in thymic epithelial cells is essential for postnatal maintenance of thymic architecture and thymocyte survival. Immunity. 2001. 15: 261-73.
84. Sano, S., et al. Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis. Embo J. 1999. 18: 4657-68.
85. Chapman, R.S., et al. Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. Genes Dev. 1999. 13: 2604-16.
86. Kaplan, M.H., et al. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. Nature. 1996. 382: 174-7.
87. Thierfelder, W.E., et al. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. Nature. 1996. 382: 171-4.
88. Liu, X., et al. Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev. 1997. 11: 179-86.
89. Feldman, G.M., et al. STAT5A-deficient mice demonstrate a defect in granulocyte-macrophage colony-stimulating factor-induced proliferation and gene expression. Blood. 1997. 90: 1768-76.
90. Udy, G.B., et al. Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. Proc Natl Acad Sci U S A. 1997. 94: 7239-44.
91. Moriggl, R., et al. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity. 1999. 10: 249-59.
92. Socolovsky, M., et al. Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell. 1999. 98: 181-91.
93. Bradley, H.L., et al. Cell intrinsic defects in cytokine responsiveness of STAT5-deficient hematopoietic stem cells. Blood. 2002. 100: 3983-9.
94. Bunting, K.D., et al. Reduced lymphomyeloid repopulating activity from adult bone marrow and fetal liver of mice lacking expression of STAT5. Blood. 2002. 99: 479-87.
95. Kieslinger, M., et al. Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation. Genes Dev. 2000. 14: 232-44.
96. Sexl, V., et al. Stat5a/b contribute to interleukin 7-induced B-cell precursor expansion, but abl- and bcr/abl-induced transformation are independent of stat5. Blood. 2000. 96: 2277-83.

97. Shimoda, K., et al. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. Nature. 1996. 380: 630-3.
98. Takeda, K., et al. Essential role of Stat6 in IL-4 signalling. Nature. 1996. 380: 627-30.
99. Kaplan, M.H., et al. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity. 1996. 4: 313-9.
100. Zhu, J., et al. Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion. J Immunol. 2001. 166: 7276-81.
101. Takeda, K., et al. Impaired IL-13-mediated functions of macrophages in STAT6-deficient mice. J Immunol. 1996. 157: 3220-2.
102. Benekli, M., et al. Signal transducer and activator of transcription proteins in leukemias. Blood. 2003. 101: 2940-54.
103. Macchi, P., et al. Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). Nature. 1995. 377: 65-8.
104. Russell, S.M., et al. Interaction of IL-2R beta and gamma c chains with Jak1 and Jak3: implications for XSCID and XCID. Science. 1994. 266: 1042-5.
105. Russell, S.M., et al. Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. Science. 1995. 270: 797-800.
106. Peschon, J.J., et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. J Exp Med. 1994. 180: 1955-60.
107. Puel, A., et al. Defective IL7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. Nat Genet. 1998. 20: 394-7.
108. Grossman, W.J., et al. Dysregulated myelopoiesis in mice lacking Jak3. Blood. 1999. 94: 932-9.
109. Kaplan, D.H., et al. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci U S A. 1998. 95: 7556-61.
110. Ramana, C.V., et al. Regulation of c-myc expression by IFN-gamma through Stat1-dependent and -independent pathways. Embo J. 2000. 19: 263-72.
111. Bromberg, J.F., et al. Stat3 as an oncogene. Cell. 1999. 98: 295-303.
112. Schwaller, J., et al. Stat5 is essential for the myelo- and lymphoproliferative disease induced by TEL/JAK2. Mol Cell. 2000. 6: 693-704.
113. Alonso, A., et al. Protein tyrosine phosphatases in the human genome. Cell. 2004. 117: 699-711.
114. Tonks, N.K. and B.G. Neel. Combinatorial control of the specificity of protein tyrosine phosphatases. Curr Opin Cell Biol. 2001. 13: 182-95.
115. Neel, B.G. and N.K. Tonks. Protein tyrosine phosphatases in signal transduction. Curr Opin Cell Biol. 1997. 9: 193-204.
116. Andersen, J.N., et al. A genomic perspective on protein tyrosine phosphatases: gene structure, pseudogenes, and genetic disease linkage. Faseb J. 2004. 18: 8-30.
117. Flint, A.J., et al. Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. Proc Natl Acad Sci U S A. 1997. 94: 1680-5.
118. Mustelin, T., et al. Protein tyrosine phosphatases and the immune response. Nat Rev Immunol. 2005. 5: 43-57.

119. Klingmuller, U., et al. Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. Cell. 1995. 80: 729-38.
120. Irie-Sasaki, J., et al. CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling. Nature. 2001. 409: 349-54.
121. Yamada, H., et al. Enhanced generation of NK cells with intact cytotoxic function in CD45 exon 6-deficient mice. J Immunol. 1996. 157: 1523-8.
122. Fleming, H.E., et al. CD45-deficient mice accumulate Pro-B cells both in vivo and in vitro. J Immunol. 2004. 173: 2542-51.
123. Tanuma, N., et al. Protein-tyrosine phosphatase PTPepsilon C inhibits Jak-STAT signaling and differentiation induced by interleukin-6 and leukemia inhibitory factor in M1 leukemia cells. J Biol Chem. 2000. 275: 28216-21.
124. Tanuma, N., et al. Protein tyrosine phosphatase epsilonC selectively inhibits interleukin-6- and interleukin- 10-induced JAK-STAT signaling. Blood. 2001. 98: 3030-4.
125. Zabolotny, J.M., et al. PTP1B regulates leptin signal transduction in vivo. Dev Cell. 2002. 2: 489-95.
126. Aoki, N. and T. Matsuda. A cytosolic protein-tyrosine phosphatase PTP1B specifically dephosphorylates and deactivates prolactin-activated STAT5a and STAT5b. J Biol Chem. 2000. 275: 39718-26.
127. Cohen, J., et al. Protein tyrosine phosphatase 1B participates in the down-regulation of erythropoietin receptor signalling. Biochem J. 2004. 377: 517-24.
128. ten Hoeve, J., et al. Identification of a nuclear Stat1 protein tyrosine phosphatase. Mol Cell Biol. 2002. 22: 5662-8.
129. Yamamoto, T., et al. The nuclear isoform of protein-tyrosine phosphatase TC-PTP regulates interleukin-6-mediated signaling pathway through STAT3 dephosphorylation. Biochem Biophys Res Commun. 2002. 297: 811-7.
130. Aoki, N. and T. Matsuda. A nuclear protein tyrosine phosphatase TC-PTP is a potential negative regulator of the PRL-mediated signaling pathway: dephosphorylation and deactivation of signal transducer and activator of transcription 5a and 5b by TC-PTP in nucleus. Mol Endocrinol. 2002. 16: 58-69.
131. Simoncic, P.D., et al. The T cell protein tyrosine phosphatase is a negative regulator of janus family kinases 1 and 3. Curr Biol. 2002. 12: 446-53.
132. David, M., et al. Differential regulation of the alpha/beta interferon-stimulated Jak/Stat pathway by the SH2 domain-containing tyrosine phosphatase SHPTP1. Mol Cell Biol. 1995. 15: 7050-8.
133. Hackett, R.H., et al. Mapping of a cytoplasmic domain of the human growth hormone receptor that regulates rates of inactivation of Jak2 and Stat proteins. J Biol Chem. 1997. 272: 11128-32.
134. Haque, S.J., et al. Protein-tyrosine phosphatase Shp-1 is a negative regulator of IL-4- and IL-13-dependent signal transduction. J Biol Chem. 1998. 273: 33893-6.
135. Yi, T., et al. Hematopoietic cell phosphatase associates with the interleukin-3 (IL-3) receptor beta chain and down-regulates IL-3-induced tyrosine phosphorylation and mitogenesis. Mol Cell Biol. 1993. 13: 7577-86.
136. Lehmann, U., et al. SHP2 and SOCS3 contribute to Tyr-759-dependent attenuation of interleukin-6 signaling through gp130. J Biol Chem. 2003. 278: 661-71.

137. Wu, T.R., et al. SHP-2 is a dual-specificity phosphatase involved in Stat1 dephosphorylation at both tyrosine and serine residues in nuclei. J Biol Chem. 2002. 277: 47572-80.
138. Du, Z., et al. Inhibition of IFN-alpha signaling by a PKC- and protein tyrosine phosphatase SHP-2-dependent pathway. Proc Natl Acad Sci U S A. 2005. 102: 10267-72.
139. Ilangumaran, S., et al. Regulation of the immune system by SOCS family adaptor proteins. Semin Immunol. 2004. 16: 351-65.
140. Elliott, J. and J.A. Johnston. SOCS: role in inflammation, allergy and homeostasis. Trends Immunol. 2004. 25: 434-40.
141. Alexander, W.S. Suppressors of cytokine signalling (SOCS) in the immune system. Nat Rev Immunol. 2002. 2: 410-6.
142. Kamura, T., et al. VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases. Genes Dev. 2004. 18: 3055-65.
143. Kamura, T., et al. The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. Genes Dev. 1998. 12: 3872-81.
144. Rui, L., et al. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. J Biol Chem. 2002. 277: 42394-8.
145. Kamizono, S., et al. The SOCS box of SOCS-1 accelerates ubiquitin-dependent proteolysis of TEL-JAK2. J Biol Chem. 2001. 276: 12530-8.
146. Ungureanu, D., et al. Regulation of Jak2 through the ubiquitin-proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS-1. Mol Cell Biol. 2002. 22: 3316-26.
147. Matsumoto, A., et al. CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. Blood. 1997. 89: 3148-54.
148. Ram, P.A. and D.J. Waxman. SOCS/CIS protein inhibition of growth hormone-stimulated STAT5 signaling by multiple mechanisms. J Biol Chem. 1999. 274: 35553-61.
149. Qing, Y., et al. Role of tyrosine 441 of interferon-gamma receptor subunit 1 in SOCS-1-mediated attenuation of STAT1 activation. J Biol Chem. 2005. 280: 1849-53.
150. Yasukawa, H., et al. The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. Embo J. 1999. 18: 1309-20.
151. Shuai, K. and B. Liu. Regulation of gene-activation pathways by PIAS proteins in the immune system. Nat Rev Immunol. 2005. 5: 593-605.
152. Liu, B., et al. A transcriptional corepressor of Stat1 with an essential LXXLL signature motif. Proc Natl Acad Sci U S A. 2001. 98: 3203-7.
153. Kotaja, N., et al. PIAS proteins modulate transcription factors by functioning as SUMO-1 ligases. Mol Cell Biol. 2002. 22: 5222-34.
154. Chung, C.D., et al. Specific inhibition of Stat3 signal transduction by PIAS3. Science. 1997. 278: 1803-5.
155. Yoshimura, A., et al. A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. Embo J. 1995. 14: 2816-26.

156. Landsman, T. and D.J. Waxman. Role of the Cytokine-induced SH2 Domain-containing Protein CIS in Growth Hormone Receptor Internalization. J Biol Chem. 2005. 280: 37471-80.
157. Matsumoto, A., et al. Suppression of STAT5 functions in liver, mammary glands, and T cells in cytokine-inducible SH2-containing protein 1 transgenic mice. Mol Cell Biol. 1999. 19: 6396-407.
158. Alexander, W.S., et al. SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. Cell. 1999. 98: 597-608.
159. Starr, R., et al. Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1. Proc Natl Acad Sci U S A. 1998. 95: 14395-9.
160. Nakagawa, R., et al. SOCS-1 participates in negative regulation of LPS responses. Immunity. 2002. 17: 677-87.
161. Kinjyo, I., et al. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. Immunity. 2002. 17: 583-91.
162. Metcalf, D., et al. Gigantism in mice lacking suppressor of cytokine signalling-2. Nature. 2000. 405: 1069-73.
163. Greenhalgh, C.J., et al. SOCS2 negatively regulates growth hormone action in vitro and in vivo. J Clin Invest. 2005. 115: 397-406.
164. Roberts, A.W., et al. Placental defects and embryonic lethality in mice lacking suppressor of cytokine signaling 3. Proc Natl Acad Sci U S A. 2001. 98: 9324-9.
165. Yasukawa, H., et al. IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. Nat Immunol. 2003. 4: 551-6.
166. Lang, R., et al. SOCS3 regulates the plasticity of gp130 signaling. Nat Immunol. 2003. 4: 546-50.
167. Croker, B.A., et al. SOCS3 is a critical physiological negative regulator of G-CSF signaling and emergency granulopoiesis. Immunity. 2004. 20: 153-65.
168. Robb, L., et al. Genetic reduction of embryonic leukemia-inhibitory factor production rescues placentation in SOCS3-null embryos but does not prevent inflammatory disease. Proc Natl Acad Sci U S A. 2005. 102: 16333-8.
169. Kario, E., et al. Suppressors of cytokine signaling 4 and 5 regulate epidermal growth factor receptor signaling. J Biol Chem. 2005. 280: 7038-48.
170. Seki, Y., et al. Expression of the suppressor of cytokine signaling-5 (SOCS5) negatively regulates IL-4-dependent STAT6 activation and Th2 differentiation. Proc Natl Acad Sci U S A. 2002. 99: 13003-8.
171. Brender, C., et al. SOCS5 is expressed in primary B and T lymphoid cells but is dispensable for lymphocyte production and function. Mol Cell Biol. 2004. 24: 6094-103.
172. Nicholson, S.E., et al. Suppressor of cytokine signaling (SOCS)-5 is a potential negative regulator of epidermal growth factor signaling. Proc Natl Acad Sci U S A. 2005. 102: 2328-33.
173. Bayle, J., et al. Suppressor of cytokine signaling 6 associates with KIT and regulates KIT receptor signaling. J Biol Chem. 2004. 279: 12249-59.
174. Krebs, D.L., et al. SOCS-6 binds to insulin receptor substrate 4, and mice lacking the SOCS-6 gene exhibit mild growth retardation. Mol Cell Biol. 2002. 22: 4567-78.

175. Krebs, D.L., et al. Development of hydrocephalus in mice lacking SOCS7. Proc Natl Acad Sci U S A. 2004. 101: 15446-51.
176. Liu, B., et al. Inhibition of Stat1-mediated gene activation by PIAS1. Proc Natl Acad Sci U S A. 1998. 95: 10626-31.
177. Liu, B., et al. PIAS1 selectively inhibits interferon-inducible genes and is important in innate immunity. Nat Immunol. 2004. 5: 891-8.
178. Liu, B., et al. Negative regulation of NF-kappaB signaling by PIAS1. Mol Cell Biol. 2005. 25: 1113-23.
179. Arora, T., et al. PIASx is a transcriptional co-repressor of signal transducer and activator of transcription 4. J Biol Chem. 2003. 278: 21327-30.
180. Wong, K.A., et al. Protein inhibitor of activated STAT Y (PIASy) and a splice variant lacking exon 6 enhance sumoylation but are not essential for embryogenesis and adult life. Mol Cell Biol. 2004. 24: 5577-86.
181. Roth, W., et al. PIASy-deficient mice display modest defects in IFN and Wnt signaling. J Immunol. 2004. 173: 6189-99.
182. Tonks, N.K., et al. Demonstration that the leukocyte common antigen CD45 is a protein tyrosine phosphatase. Biochemistry. 1988. 27: 8695-701.
183. Huntington, N.D. and D.M. Tarlinton. CD45: direct and indirect government of immune regulation. Immunol Lett. 2004. 94: 167-74.
184. Byth, K.F., et al. CD45-null transgenic mice reveal a positive regulatory role for CD45 in early thymocyte development, in the selection of CD4+CD8+ thymocytes, and B cell maturation. J Exp Med. 1996. 183: 1707-18.
185. Roach, T., et al. CD45 regulates Src family member kinase activity associated with macrophage integrin-mediated adhesion. Curr Biol. 1997. 7: 408-17.
186. Nakamura, K., et al. Molecular cloning of a novel cytoplasmic protein tyrosine phosphatase PTP epsilon. Biochem Biophys Res Commun. 1996. 218: 726-32.
187. Gil-Henn, H. and A. Elson. Tyrosine phosphatase-epsilon activates Src and supports the transformed phenotype of Neu-induced mammary tumor cells. J Biol Chem. 2003. 278: 15579-86.
188. Elson, A. and P. Leder. Identification of a cytoplasmic, phorbol ester-inducible isoform of protein tyrosine phosphatase epsilon. Proc Natl Acad Sci U S A. 1995. 92: 12235-9.
189. Tanuma, N., et al. Distinct promoters control transmembrane and cytosolic protein tyrosine phosphatase epsilon expression during macrophage differentiation. Eur J Biochem. 1999. 259: 46-54.
190. Peretz, A., et al. Hypomyelination and increased activity of voltage-gated K(+) channels in mice lacking protein tyrosine phosphatase epsilon. Embo J. 2000. 19: 4036-45.
191. Chiusaroli, R., et al. Tyrosine phosphatase epsilon is a positive regulator of osteoclast function in vitro and in vivo. Mol Biol Cell. 2004. 15: 234-44.
192. Matthews, R.J., et al. Characterization of hematopoietic intracellular protein tyrosine phosphatases: description of a phosphatase containing an SH2 domain and another enriched in proline-, glutamic acid-, serine-, and threonine-rich sequences. Mol Cell Biol. 1992. 12: 2396-405.
193. Yang, J., et al. Crystal structure of the catalytic domain of protein-tyrosine phosphatase SHP-1. J Biol Chem. 1998. 273: 28199-207.

194. Shultz, L.D., et al. Mutations at the murine motheaten locus are within the hematopoietic cell protein-tyrosine phosphatase (Hcph) gene. Cell. 1993. 73: 1445-54.
195. Tsui, H.W., et al. Motheaten and viable motheaten mice have mutations in the haematopoietic cell phosphatase gene. Nat Genet. 1993. 4: 124-9.
196. Chim, C.S., et al. SOCS1 and SHP1 hypermethylation in multiple myeloma: implications for epigenetic activation of the Jak/STAT pathway. Blood. 2004. 103: 4630-5.
197. Chim, C.S., et al. Epigenetic dysregulation of the Jak/STAT pathway by frequent aberrant methylation of SHP1 but not SOCS1 in acute leukaemias. Ann Hematol. 2004. 83: 527-32.
198. Cheng, J., et al. Down-regulation of SHP1 and up-regulation of negative regulators of JAK/STAT signaling in HTLV-1 transformed cell lines and freshly transformed human peripheral blood CD4+ T-cells. Leuk Res. 2004. 28: 71-82.
199. Mena-Duran, A.V., et al. SHP1 expression in bone marrow biopsies of myelodysplastic syndrome patients: a new prognostic factor. Br J Haematol. 2005. 129: 791-4.
200. Chen, H.E., et al. Regulation of colony-stimulating factor 1 receptor signaling by the SH2 domain-containing tyrosine phosphatase SHPTP1. Mol Cell Biol. 1996. 16: 3685-97.
201. Berg, K.L., et al. SHP-1 regulation of p62(DOK) tyrosine phosphorylation in macrophages. J Biol Chem. 1999. 274: 35855-65.
202. Aoki, K., et al. The tyrosine phosphatase SHP-1 is a negative regulator of osteoclastogenesis and osteoclast resorbing activity: increased resorption and osteopenia in me(v)/me(v) mutant mice. Bone. 1999. 25: 261-7.
203. Umeda, S., et al. Deficiency of SHP-1 protein-tyrosine phosphatase activity results in heightened osteoclast function and decreased bone density. Am J Pathol. 1999. 155: 223-33.
204. Kant, A.M., et al. SHP-1 regulates Fcgamma receptor-mediated phagocytosis and the activation of RAC. Blood. 2002. 100: 1852-9.
205. Nandan, D., et al. Leishmania EF-1alpha activates the Src homology 2 domain containing tyrosine phosphatase SHP-1 leading to macrophage deactivation. J Biol Chem. 2002. 277: 50190-7.
206. Nandan, D., et al. Activation of phosphotyrosine phosphatase activity attenuates mitogen-activated protein kinase signaling and inhibits c-FOS and nitric oxide synthase expression in macrophages infected with Leishmania donovani. Infect Immun. 1999. 67: 4055-63.
207. Blanchette, J., et al. Leishmania-induced increases in activation of macrophage SHP-1 tyrosine phosphatase are associated with impaired IFN-gamma-triggered JAK2 activation. Eur J Immunol. 1999. 29: 3737-44.
208. Nandan, D. and N.E. Reiner. Attenuation of gamma interferon-induced tyrosine phosphorylation in mononuclear phagocytes infected with Leishmania donovani: selective inhibition of signaling through Janus kinases and Stat1. Infect Immun. 1995. 63: 4495-500.
209. Hanafusa, H., et al. Shp2, an SH2-containing protein-tyrosine phosphatase, positively regulates receptor tyrosine kinase signaling by dephosphorylating and inactivating the inhibitor Sprouty. J Biol Chem. 2004. 279: 22992-5.

210. Zhang, S.Q., et al. Shp2 regulates SRC family kinase activity and Ras/Erk activation by controlling Csk recruitment. Mol Cell. 2004. 13: 341-55.
211. Zhang, S.Q., et al. Receptor-specific regulation of phosphatidylinositol 3'-kinase activation by the protein tyrosine phosphatase Shp2. Mol Cell Biol. 2002. 22: 4062-72.
212. Schaper, F., et al. Activation of the protein tyrosine phosphatase SHP2 via the interleukin-6 signal transducing receptor protein gp130 requires tyrosine kinase Jak1 and limits acute-phase protein expression. Biochem J. 1998. 335 (Pt 3): 557-65.
213. Okuda, K., et al. Signaling functions of the tyrosine residues in the betac chain of the granulocyte-macrophage colony-stimulating factor receptor. Blood. 1997. 90: 4759-66.
214. Araki, T., et al. Tyrosyl phosphorylation of Shp2 is required for normal ERK activation in response to some, but not all, growth factors. J Biol Chem. 2003. 278: 41677-84.
215. Saxton, T.M. and T. Pawson. Morphogenetic movements at gastrulation require the SH2 tyrosine phosphatase Shp2. Proc Natl Acad Sci U S A. 1999. 96: 3790-5.
216. Saxton, T.M., et al. The SH2 tyrosine phosphatase shp2 is required for mammalian limb development. Nat Genet. 2000. 24: 420-3.
217. Tartaglia, M., et al. Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. Nat Genet. 2001. 29: 465-8.
218. Araki, T., et al. Mouse model of Noonan syndrome reveals cell type- and gene dosage-dependent effects of Ptpn11 mutation. Nat Med. 2004. 10: 849-57.
219. Bentires-Alj, M., et al. Activating mutations of the noonan syndrome-associated SHP2/PTPN11 gene in human solid tumors and adult acute myelogenous leukemia. Cancer Res. 2004. 64: 8816-20.
220. Mohi, M.G., et al. Prognostic, therapeutic, and mechanistic implications of a mouse model of leukemia evoked by Shp2 (PTPN11) mutations. Cancer Cell. 2005. 7: 179-91.
221. Cool, D.E., et al. cDNA isolated from a human T-cell library encodes a member of the protein-tyrosine-phosphatase family. Proc Natl Acad Sci U S A. 1989. 86: 5257-61.
222. Mosinger, B., Jr., et al. Cloning and characterization of a mouse cDNA encoding a cytoplasmic protein-tyrosine-phosphatase. Proc Natl Acad Sci U S A. 1992. 89: 499-503.
223. Lorenzen, J.A., et al. COOH-terminal sequence motifs target the T cell protein tyrosine phosphatase to the ER and nucleus. J Cell Biol. 1995. 131: 631-43.
224. Tillmann, U., et al. Nuclear localization and cell cycle regulation of a murine protein tyrosine phosphatase. Mol Cell Biol. 1994. 14: 3030-40.
225. Tiganis, T., et al. Association of the T-cell protein tyrosine phosphatase with nuclear import factor p97. J Biol Chem. 1997. 272: 21548-57.
226. Reddy, R.S. and G. Swarup. Alternative splicing generates four different forms of a non-transmembrane protein tyrosine phosphatase mRNA. DNA Cell Biol. 1995. 14: 1007-15.
227. Tiganis, T., et al. Epidermal growth factor receptor and the adaptor protein p52Shc are specific substrates of T-cell protein tyrosine phosphatase. Mol Cell Biol. 1998. 18: 1622-34.

228. Tiganis, T., et al. The protein-tyrosine phosphatase TCPTP regulates epidermal growth factor receptor-mediated and phosphatidylinositol 3-kinase-dependent signaling. J Biol Chem. 1999. 274: 27768-75.
229. Lam, M.H., et al. Cellular stress regulates the nucleocytoplasmic distribution of the protein-tyrosine phosphatase TCPTP. J Biol Chem. 2001. 276: 37700-7.
230. Mattila, E., et al. Negative regulation of EGFR signalling through integrin-alpha1beta1-mediated activation of protein tyrosine phosphatase TCPTP. Nat Cell Biol. 2005. 7: 78-85.
231. Klingler-Hoffmann, M., et al. The protein tyrosine phosphatase TCPTP suppresses the tumorigenicity of glioblastoma cells expressing a mutant epidermal growth factor receptor. J Biol Chem. 2001. 276: 46313-8.
232. Persson, C., et al. Site-selective regulation of platelet-derived growth factor beta receptor tyrosine phosphorylation by T-cell protein tyrosine phosphatase. Mol Cell Biol. 2004. 24: 2190-201.
233. Galic, S., et al. Coordinated regulation of insulin signaling by the protein tyrosine phosphatases PTP1B and TCPTP. Mol Cell Biol. 2005. 25: 819-29.
234. Galic, S., et al. Regulation of insulin receptor signaling by the protein tyrosine phosphatase TCPTP. Mol Cell Biol. 2003. 23: 2096-108.
235. Pasquali, C., et al. Identification of protein tyrosine phosphatases with specificity for the ligand-activated growth hormone receptor. Mol Endocrinol. 2003. 17: 2228-39.
236. Frangioni, J.V., et al. The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. Cell. 1992. 68: 545-60.
237. Barford, D., et al. Crystal structure of human protein tyrosine phosphatase 1B. Science. 1994. 263: 1397-404.
238. Flint, A.J., et al. Multi-site phosphorylation of the protein tyrosine phosphatase, PTP1B: identification of cell cycle regulated and phorbol ester stimulated sites of phosphorylation. Embo J. 1993. 12: 1937-46.
239. Frangioni, J.V., et al. Calpain-catalyzed cleavage and subcellular relocation of protein phosphotyrosine phosphatase 1B (PTP-1B) in human platelets. Embo J. 1993. 12: 4843-56.
240. Milarski, K.L., et al. Sequence specificity in recognition of the epidermal growth factor receptor by protein tyrosine phosphatase 1B. J Biol Chem. 1993. 268: 23634-9.
241. Liu, F. and J. Chernoff. Protein tyrosine phosphatase 1B interacts with and is tyrosine phosphorylated by the epidermal growth factor receptor. Biochem J. 1997. 327 (Pt 1): 139-45.
242. Haj, F.G., et al. Regulation of receptor tyrosine kinase signaling by protein tyrosine phosphatase-1B. J Biol Chem. 2003. 278: 739-44.
243. Dube, N., et al. The role of protein tyrosine phosphatase 1B in Ras signaling. Proc Natl Acad Sci U S A. 2004. 101: 1834-9.
244. Seely, B.L., et al. Protein tyrosine phosphatase 1B interacts with the activated insulin receptor. Diabetes. 1996. 45: 1379-85.
245. Klaman, L.D., et al. Increased energy expenditure, decreased adiposity, and tissue-specific insulin sensitivity in protein-tyrosine phosphatase 1B-deficient mice. Mol Cell Biol. 2000. 20: 5479-89.

246. Buckley, D.A., et al. Regulation of insulin-like growth factor type I (IGF-I) receptor kinase activity by protein tyrosine phosphatase 1B (PTP-1B) and enhanced IGF-I-mediated suppression of apoptosis and motility in PTP-1B-deficient fibroblasts. Mol Cell Biol. 2002. 22: 1998-2010.
247. Buckley, D.A., et al. Identification of an IGF-1R kinase regulatory phosphatase using the fission yeast Schizosaccharomyces pombe and a GFP tagged IGF-1R in mammalian cells. Mol Pathol. 2002. 55: 46-54.
248. Gu, F., et al. Protein-tyrosine phosphatase 1B potentiates IRE1 signaling during endoplasmic reticulum stress. J Biol Chem. 2004. 279: 49689-93.
249. Cheng, A., et al. Attenuation of adhesion-dependent signaling and cell spreading in transformed fibroblasts lacking protein tyrosine phosphatase-1B. J Biol Chem. 2001. 276: 25848-55.
250. Zhang, Z., et al. Phosphorylated alpha -actinin and PTP 1B coregulate the disassembly of the FAK/Src complex and promote cell migration. J Biol Chem. 2005.
251. Liu, F., et al. Protein tyrosine phosphatase 1B negatively regulates integrin signaling. Curr Biol. 1998. 8: 173-6.
252. LaMontagne, K.R., Jr., et al. Protein tyrosine phosphatase 1B antagonizes signalling by oncoprotein tyrosine kinase p210 bcr-abl in vivo. Mol Cell Biol. 1998. 18: 2965-75.
253. Ibarra-Sanchez, M.J., et al. The T-cell protein tyrosine phosphatase. Semin Immunol. 2000. 12: 379-86.
254. Dupuis, M., et al. Gr-1+ myeloid cells lacking T cell protein tyrosine phosphatase inhibit lymphocyte proliferation by an IFN-gamma- and nitric oxide-dependent mechanism. J Immunol. 2003. 171: 726-32.
255. Zabolotny, J.M., et al. Transgenic overexpression of protein-tyrosine phosphatase 1B in muscle causes insulin resistance, but overexpression with leukocyte antigen-related phosphatase does not additively impair insulin action. J Biol Chem. 2004. 279: 24844-51.
256. Haj, F.G., et al. Liver-specific protein-tyrosine phosphatase 1B (PTP1B) re-expression alters glucose homeostasis of PTP1B-/-mice. J Biol Chem. 2005. 280: 15038-46.
257. Johnson, T.O., et al. Protein tyrosine phosphatase 1B inhibitors for diabetes. Nat Rev Drug Discov. 2002. 1: 696-709.
258. Harley, E.A. and N. Levens. Protein tyrosine phosphatase 1B inhibitors for the treatment of type 2 diabetes and obesity: recent advances. Curr Opin Investig Drugs. 2003. 4: 1179-89.
259. Rondinone, C.M., et al. Protein tyrosine phosphatase 1B reduction regulates adiposity and expression of genes involved in lipogenesis. Diabetes. 2002. 51: 2405-11.
260. Gum, R.J., et al. Reduction of protein tyrosine phosphatase 1B increases insulin-dependent signaling in ob/ob mice. Diabetes. 2003. 52: 21-8.
261. Palmer, N.D., et al. Association of protein tyrosine phosphatase 1B gene polymorphisms with measures of glucose homeostasis in Hispanic Americans: the insulin resistance atherosclerosis study (IRAS) family study. Diabetes. 2004. 53: 3013-9.

262. Bento, J.L., et al. Association of protein tyrosine phosphatase 1B gene polymorphisms with type 2 diabetes. Diabetes. 2004. 53: 3007-12.
263. Florez, J.C., et al. Association testing of the protein tyrosine phosphatase 1B gene (PTPN1) with type 2 diabetes in 7,883 people. Diabetes. 2005. 54: 1884-91.
264. Nathan, C. Points of control in inflammation. Nature. 2002. 420: 846-52.
265. Gifford, G.E. and M.L. Lohmann-Matthes. Gamma interferon priming of mouse and human macrophages for induction of tumor necrosis factor production by bacterial lipopolysaccharide. J Natl Cancer Inst. 1987. 78: 121-4.
266. Ding, A.H., et al. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J Immunol. 1988. 141: 2407-12.
267. Mannel, D.N., et al. Macrophages as a source of tumoricidal activity (tumor-necrotizing factor). Infect Immun. 1980. 30: 523-30.
268. Stuehr, D.J. and M.A. Marletta. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-gamma. J Immunol. 1987. 139: 518-25.
269. Mohan, K., et al. CXCR3 is required for migration to dermal inflammation by normal and in vivo activated T cells: differential requirements by CD4 and CD8 memory subsets. Eur J Immunol. 2005. 35: 1702-11.
270. Adair-Kirk, T.L., et al. A chemotactic peptide from laminin alpha 5 functions as a regulator of inflammatory immune responses via TNF alpha-mediated signaling. J Immunol. 2005. 174: 1621-9.
271. Tracey, K.J., et al. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature. 1987. 330: 662-4.
272. Tracey, K.J., et al. Cachectin/tumor necrosis factor induces cachexia, anemia, and inflammation. J Exp Med. 1988. 167: 1211-27.
273. Ashkenazi, A., et al. Protection against endotoxic shock by a tumor necrosis factor receptor immunoadhesin. Proc Natl Acad Sci U S A. 1991. 88: 10535-9.
274. Kontoyiannis, D., et al. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. Immunity. 1999. 10: 387-98.
275. Hanauer, S.B., et al. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. Lancet. 2002. 359: 1541-9.
276. Targan, S.R., et al. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. N Engl J Med. 1997. 337: 1029-35.
277. Liu, J.S., et al. Expression of inducible nitric oxide synthase and nitrotyrosine in multiple sclerosis lesions. Am J Pathol. 2001. 158: 2057-66.
278. Bogdan, C. Nitric oxide and the immune response. Nat Immunol. 2001. 2: 907-16.
279. Fehsel, K., et al. Nitric oxide induces apoptosis in mouse thymocytes. J Immunol. 1995. 155: 2858-65.
280. Tai, X.G., et al. Expression of an inducible type of nitric oxide (NO) synthase in the thymus and involvement of NO in deletion of TCR-stimulated double-positive thymocytes. J Immunol. 1997. 158: 4696-703.
281. Bobe, P., et al. Nitric oxide mediation of active immunosuppression associated with graft-versus-host reaction. Blood. 1999. 94: 1028-37.

282. Robinson, V.K., et al. Peroxynitrite inhibits inducible (type 2) nitric oxide synthase in murine lung epithelial cells in vitro. Free Radic Biol Med. 2001. 30: 986-91.
283. Niinobu, T., et al. Negative feedback regulation of activated macrophages via Fas-mediated apoptosis. Am J Physiol Cell Physiol. 2000. 279: C504-9.
284. Albina, J.E., et al. Nitric oxide-mediated apoptosis in murine peritoneal macrophages. J Immunol. 1993. 150: 5080-5.
285. Cohen, J. The immunopathogenesis of sepsis. Nature. 2002. 420: 885-91.
286. Nestel, F.P., et al. Macrophage priming and lipopolysaccharide-triggered release of tumor necrosis factor alpha during graft-versus-host disease. J Exp Med. 1992. 175: 405-13.
287. Vaishnaw, A.K., et al. The spectrum of apoptotic defects and clinical manifestations, including systemic lupus erythematosus, in humans with CD95 (Fas/APO-1) mutations. Arthritis Rheum. 1999. 42: 1833-42.
288. Watanabe-Fukunaga, R., et al. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature. 1992. 356: 314-7.
289. Rieux-Laucat, F., et al. Cell-death signaling and human disease. Curr Opin Immunol. 2003. 15: 325-31.
290. Walport, M.J., et al. Complement deficiency and autoimmunity. Ann N Y Acad Sci. 1997. 815: 267-81.
291. Botto, M., et al. Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nat Genet. 1998. 19: 56-9.
292. Kulkarni, A.B., et al. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci U S A. 1993. 90: 770-4.
293. Shull, M.M., et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature. 1992. 359: 693-9.
294. Ueda, H., et al. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature. 2003. 423: 506-11.
295. Tivol, E.A., et al. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity. 1995. 3: 541-7.
296. Waterhouse, P., et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science. 1995. 270: 985-8.
297. Bachmaier, K., et al. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. Nature. 2000. 403: 211-6.
298. Weih, F., et al. Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kappa B/Rel family. Cell. 1995. 80: 331-40.
299. Shultz, L.D., et al. Hematopoietic stem cell function in motheaten mice. Exp Hematol. 1983. 11: 667-80.
300. Nestel, F.P., et al. Activation of macrophage cytostatic effector mechanisms during acute graft-versus-host disease: release of intracellular iron and nitric oxide-mediated cytostasis. Blood. 2000. 96: 1836-43.
301. Heinonen, K.M., et al. T-cell protein tyrosine phosphatase deletion results in progressive systemic inflammatory disease. Blood. 2004. 103: 3457-64.

302. Akashi, K., et al. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature. 2000. 404: 193-7.
303. Friedman, A.D. Transcriptional regulation of granulocyte and monocyte development. Oncogene. 2002. 21: 3377-90.
304. Stanley, E., et al. Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. Proc Natl Acad Sci U S A. 1994. 91: 5592-6.
305. Lieschke, G.J., et al. Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. Blood. 1994. 84: 1737-46.
306. Seymour, J.F., et al. Mice lacking both granulocyte colony-stimulating factor (CSF) and granulocyte-macrophage CSF have impaired reproductive capacity, perturbed neonatal granulopoiesis, lung disease, amyloidosis, and reduced long-term survival. Blood. 1997. 90: 3037-49.
307. Wiktor-Jedrzejczak, W.W., et al. Hematological characterization of congenital osteopetrosis in op/op mouse. Possible mechanism for abnormal macrophage differentiation. J Exp Med. 1982. 156: 1516-27.
308. Wiktor-Jedrzejczak, W., et al. Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. Proc Natl Acad Sci U S A. 1990. 87: 4828-32.
309. Lieschke, G.J., et al. Mice lacking both macrophage- and granulocyte-macrophage colony-stimulating factor have macrophages and coexistent osteopetrosis and severe lung disease. Blood. 1994. 84: 27-35.
310. Rosmarin, A.G., et al. Transcriptional regulation in myelopoiesis: Hematopoietic fate choice, myeloid differentiation, and leukemogenesis. Exp Hematol. 2005. 33: 131-43.
311. Scott, E.W., et al. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science. 1994. 265: 1573-7.
312. McKercher, S.R., et al. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. Embo J. 1996. 15: 5647-58.
313. Anderson, K.L., et al. Myeloid development is selectively disrupted in PU.1 null mice. Blood. 1998. 91: 3702-10.
314. McIvor, Z., et al. Transient expression of PU.1 commits multipotent progenitors to a myeloid fate whereas continued expression favors macrophage over granulocyte differentiation. Exp Hematol. 2003. 31: 39-47.
315. Dahl, R., et al. Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. Nat Immunol. 2003. 4: 1029-36.
316. Zhang, D.E., et al. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. Proc Natl Acad Sci U S A. 1997. 94: 569-74.
317. Pabst, T., et al. Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. Nat Genet. 2001. 27: 263-70.
318. Kharbanda, S., et al. Expression of the early growth response 1 and 2 zinc finger genes during induction of monocytic differentiation. J Clin Invest. 1991. 88: 571-7.

319. Tsujimura, H., et al. IFN consensus sequence binding protein/IFN regulatory factor-8 guides bone marrow progenitor cells toward the macrophage lineage. J Immunol. 2002. 169: 1261-9.
320. Krishnaraju, K., et al. Early growth response gene 1 stimulates development of hematopoietic progenitor cells along the macrophage lineage at the expense of the granulocyte and erythroid lineages. Blood. 2001. 97: 1298-305.
321. Shafarenko, M., et al. Egr-1 abrogates the block imparted by c-Myc on terminal M1 myeloid differentiation. Blood. 2005. 106: 871-8.
322. Meraro, D., et al. IFN-stimulated gene 15 is synergistically activated through interactions between the myelocyte/lymphocyte-specific transcription factors, PU.1, IFN regulatory factor-8/IFN consensus sequence binding protein, and IFN regulatory factor-4: characterization of a new subtype of IFN-stimulated response element. J Immunol. 2002. 168: 6224-31.
323. Holtschke, T., et al. Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene. Cell. 1996. 87: 307-17.
324. Yi, T., et al. Identification of novel protein tyrosine phosphatases of hematopoietic cells by polymerase chain reaction amplification. Blood. 1991. 78: 2222-8.
325. Adachi, M., et al. Protein-tyrosine phosphatase expression in pre-B cell NALM-6. Cancer Res. 1992. 52: 737-40.
326. LaMontagne, K.R., Jr., et al. Protein tyrosine phosphatase PTP1B suppresses p210 bcr-abl-induced transformation of rat-1 fibroblasts and promotes differentiation of K562 cells. Proc Natl Acad Sci U S A. 1998. 95: 14094-9.
327. Dube, N., et al. Genetic ablation of protein tyrosine phosphatase 1B accelerates lymphomagenesis of p53-null mice through the regulation of B-cell development. Cancer Res. 2005. 65: 10088-95.
328. Asante-Appiah, E., et al. The YRD motif is a major determinant of substrate and inhibitor specificity in T-cell protein-tyrosine phosphatase. J Biol Chem. 2001. 276: 26036-43.
329. Romsicki, Y., et al. Purification and characterization of T cell protein tyrosine phosphatase reveals significant functional homology to protein tyrosine phosphatase-1B. Arch Biochem Biophys. 2003. 414: 40-50.
330. Gu, D., et al. Myasthenia gravis-like syndrome induced by expression of interferon gamma in the neuromuscular junction. J Exp Med. 1995. 181: 547-57.
331. Seery, J.P., et al. Antinuclear autoantibodies and lupus nephritis in transgenic mice expressing interferon gamma in the epidermis. J Exp Med. 1997. 186: 1451-9.
332. Sarvetnick, N., et al. Loss of pancreatic islet tolerance induced by beta-cell expression of interferon-gamma. Nature. 1990. 346: 844-7.
333. Toyonaga, T., et al. Chronic active hepatitis in transgenic mice expressing interferon-gamma in the liver. Proc Natl Acad Sci U S A. 1994. 91: 614-8.
334. Akwa, Y., et al. Transgenic expression of IFN-alpha in the central nervous system of mice protects against lethal neurotropic viral infection but induces inflammation and neurodegeneration. J Immunol. 1998. 161: 5016-26.
335. Stewart, T.A., et al. Induction of type I diabetes by interferon-alpha in transgenic mice. Science. 1993. 260: 1942-6.

336. Malik, N., et al. Developmental abnormalities in mice transgenic for bovine oncostatin M. Mol Cell Biol. 1995. 15: 2349-58.
337. Clegg, C.H., et al. Oncostatin M transforms lymphoid tissue function in transgenic mice by stimulating lymph node T-cell development and thymus autoantibody production. Exp Hematol. 1999. 27: 712-25.
338. Shen, M.M., et al. Expression of LIF in transgenic mice results in altered thymic epithelium and apparent interconversion of thymic and lymph node morphologies. Embo J. 1994. 13: 1375-85.
339. Suematsu, S., et al. IgG1 plasmacytosis in interleukin 6 transgenic mice. Proc Natl Acad Sci U S A. 1989. 86: 7547-51.
340. Lee, M.S., et al. Pancreatic islet production of murine interleukin-10 does not inhibit immune-mediated tissue destruction. J Clin Invest. 1994. 93: 1332-8.
341. Lee, M.S., et al. IL-10 is necessary and sufficient for autoimmune diabetes in conjunction with NOD MHC homozygosity. J Exp Med. 1996. 183: 2663-8.
342. Wogensen, L., et al. Production of interleukin 10 by islet cells accelerates immune-mediated destruction of beta cells in nonobese diabetic mice. J Exp Med. 1994. 179: 1379-84.
343. Hagenbaugh, A., et al. Altered immune responses in interleukin 10 transgenic mice. J Exp Med. 1997. 185: 2101-10.
344. Kichian, K., et al. IL-12 p40 messenger RNA expression in target organs during acute graft-versus-host disease. Possible involvement of IFN-gamma. J Immunol. 1996. 157: 2851-6.
345. Price, K.S., et al. Progressive accumulation of bacterial lipopolysaccharide in vivo during murine acute graft-versus-host disease. Scand J Immunol. 1997. 45: 294-300.
346. Garside, P., et al. Nitric oxide mediates intestinal pathology in graft-vs.-host disease. Eur J Immunol. 1992. 22: 2141-5.
347. Dalton, D.K., et al. Interferon gamma eliminates responding CD4 T cells during mycobacterial infection by inducing apoptosis of activated CD4 T cells. J Exp Med. 2000. 192: 117-22.
348. Angulo, I., et al. Nitric oxide-producing CD11b(+)Ly-6G(Gr-1)(+)CD31(ER-MP12)(+) cells in the spleen of cyclophosphamide-treated mice: implications for T-cell responses in immunosuppressed mice. Blood. 2000. 95: 212-20.
349. McCormack, J.M., et al. Macrophage progenitors from mouse bone marrow and spleen differ in their expression of the Ly-6C differentiation antigen. J Immunol. 1993. 151: 6389-98.
350. Schoenhaut, D.S., et al. Cloning and expression of murine IL-12. J Immunol. 1992. 148: 3433-40.
351. Howard, J.G. Increased sensitivity to bacterial endotoxin of F-1 hybrid mice undergoing graft-versus-host reaction. Nature. 1961. 190: 1122.
352. Katschinski, T., et al. Gamma interferon mediates Propionibacterium acnes-induced hypersensitivity to lipopolysaccharide in mice. Infect Immun. 1992. 60: 1994-2001.
353. Dinges, M.M. and P.M. Schlievert. Role of T cells and gamma interferon during induction of hypersensitivity to lipopolysaccharide by toxic shock syndrome toxin 1 in mice. Infect Immun. 2001. 69: 1256-64.

354. Read, T.E., et al. Chylomicrons enhance endotoxin excretion in bile. Infect Immun. 1993. 61: 3496-502.
355. Cooke, K.R., et al. LPS antagonism reduces graft-versus-host disease and preserves graft-versus-leukemia activity after experimental bone marrow transplantation. J Clin Invest. 2001. 107: 1581-9.
356. Cooke, K.R., et al. The role of endotoxin and the innate immune response in the pathophysiology of acute graft versus host disease. J Endotoxin Res. 2002. 8: 441-8.
357. Teshima, T., et al. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. Nat Med. 2002. 8: 575-81.
358. Okamura, H., et al. Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature. 1995. 378: 88-91.
359. Dieli, F., et al. Resistance of natural killer T cell-deficient mice to systemic Shwartzman reaction. J Exp Med. 2000. 192: 1645-52.
360. Ramarathinam, L., et al. Salmonella typhimurium induces IFN-gamma production in murine splenocytes. Role of natural killer cells and macrophages. J Immunol. 1993. 150: 3973-81.
361. Starr, R., et al. A family of cytokine-inducible inhibitors of signalling. Nature. 1997. 387: 917-21.
362. Wee, C., et al. Promoter analysis of the murine T-cell protein tyrosine phosphatase gene. Gene. 1999. 237: 351-60.
363. Silvennoinen, O., et al. Structure of the murine Jak2 protein-tyrosine kinase and its role in interleukin 3 signal transduction. Proc Natl Acad Sci U S A. 1993. 90: 8429-33.
364. Shimoda, K., et al. G-CSF induces tyrosine phosphorylation of the JAK2 protein in the human myeloid G-CSF responsive and proliferative cells, but not in mature neutrophils. Biochem Biophys Res Commun. 1994. 203: 922-8.
365. Quelle, F.W., et al. JAK2 associates with the beta c chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. Mol Cell Biol. 1994. 14: 4335-41.
366. Novak, U., et al. Colony-stimulating factor 1-induced STAT1 and STAT3 activation is accompanied by phosphorylation of Tyk2 in macrophages and Tyk2 and JAK1 in fibroblasts. Blood. 1995. 86: 2948-56.
367. Donato, N.J., et al. Down-regulation of interleukin-3/granulocyte-macrophage colony-stimulating factor receptor beta-chain in BCR-ABL(+) human leukemic cells: association with loss of cytokine-mediated Stat-5 activation and protection from apoptosis after BCR-ABL inhibition. Blood. 2001. 97: 2846-53.
368. Angulo, R. and D.A. Fulcher. Measurement of Candida-specific blastogenesis: comparison of carboxyfluorescein succinimidyl ester labelling of T cells, thymidine incorporation, and CD69 expression. Cytometry. 1998. 34: 143-51.
369. Passegue, E., et al. JunB deficiency leads to a myeloproliferative disorder arising from hematopoietic stem cells. Cell. 2004. 119: 431-43.
370. Rutherford, M.S. and L.B. Schook. Differential immunocompetence of macrophages derived using macrophage or granulocyte-macrophage colony-stimulating factor. J Leukoc Biol. 1992. 51: 69-76.

371. Sweet, M.J., et al. Colony-stimulating factor-1 suppresses responses to CpG DNA and expression of toll-like receptor 9 but enhances responses to lipopolysaccharide in murine macrophages. J Immunol. 2002. 168: 392-9.
372. Faggioni, R., et al. Reduced leptin levels in starvation increase susceptibility to endotoxic shock. Am J Pathol. 2000. 156: 1781-7.
373. Schmidt-Arras, D.E., et al. Tyrosine phosphorylation regulates maturation of receptor tyrosine kinases. Mol Cell Biol. 2005. 25: 3690-703.
374. Balbo, P., et al. Differential role of CD80 and CD86 on alveolar macrophages in the presentation of allergen to T lymphocytes in asthma. Clin Exp Allergy. 2001. 31: 625-36.
375. Burastero, S.E., et al. Increased expression of the CD80 accessory molecule by alveolar macrophages in asthmatic subjects and its functional involvement in allergen presentation to autologous TH2 lymphocytes. J Allergy Clin Immunol. 1999. 103: 1136-42.
376. Cheng, X., et al. CD80, but not CD86 were up-regulated on the spleen-derived dendritic cells from OVA-sensitized and challenged BALB/c mice. Immunol Lett. 2003. 89: 31-8.
377. Sangwan, V., et al. Protein tyrosine phosphatase 1B deficiency protects against fas-induced hepatic failure. J Biol Chem. 2005.
378. DeKoter, R.P., et al. PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors. Embo J. 1998. 17: 4456-68.
379. Fukada, T. and N.K. Tonks. The reciprocal role of Egr-1 and Sp family proteins in regulation of the PTP1B promoter in response to the p210 Bcr-Abl oncoprotein-tyrosine kinase. J Biol Chem. 2001. 276: 25512-9.
380. Eichhorst, S.T., et al. Suramin inhibits death receptor-induced apoptosis in vitro and fulminant apoptotic liver damage in mice. Nat Med. 2004. 10: 602-9.
381. Shimizu, T., et al. STI571-resistant KT-1 cells are sensitive to interferon-alpha accompanied by the loss of T-cell protein tyrosine phosphatase and prolonged phosphorylation of Stat1. Exp Hematol. 2003. 31: 601-8.
382. Heinonen, K.M., et al. Protein tyrosine phosphatase 1B negatively regulates macrophage development through CSF-1 signaling. Proc Natl Acad Sci U S A. 2006. 103: 2776-81.
383. Simoncic, P.D., et al. T-cell protein tyrosine phosphatase (TC-PTP) is a critical regulator of CSF-1 dependent macrophage differentiation. Mol Cell Biol. 2006.
384. McCartney-Francis, N.L., et al. Lacrimal gland inflammation is responsible for ocular pathology in TGF-beta 1 null mice. Am J Pathol. 1997. 151: 1281-8.
385. Jabs, D.A., et al. Inflammatory mediators in autoimmune lacrimal gland disease in MRL/Mpj mice. Invest Ophthalmol Vis Sci. 2004. 45: 2293-8.
386. Franklin, R.M., et al. Ocular manifestations of graft-vs-host disease. Ophthalmology. 1983. 90: 4-13.
387. Kornbluth, M., et al. T cell subsets in the thymus of graft-versus-host immunosuppressed mice. Sensitivity of the L3T4+Lyt-2- subset to cortisone. Transplantation. 1991. 51: 262-7.
388. Desbarats, J. and W.S. Lapp. Thymic selection and thymic major histocompatibility complex class II expression are abnormal in mice undergoing graft-versus-host reactions. J Exp Med. 1993. 178: 805-14.

389. Zwijsen, A., et al. Transforming growth factor beta signalling in vitro and in vivo: activin ligand-receptor interaction, Smad5 in vasculogenesis, and repression of target genes by the deltaEF1/ZEB-related SIP1 in the vertebrate embryo. Mol Cell Endocrinol. 2001. 180: 13-24.
390. Godin, I. and A. Cumano. The hare and the tortoise: an embryonic haematopoietic race. Nat Rev Immunol. 2002. 2: 593-604.
391. Baron, M.H. Embryonic origins of mammalian hematopoiesis. Exp Hematol. 2003. 31: 1160-9.
392. Dooley, S., et al. YB-1 is the crucial mediator of antifibrotic IFN-gamma effects. J Biol Chem. 2005.
393. Higashi, K., et al. Interferon-gamma interferes with transforming growth factor-beta signaling through direct interaction of YB-1 with Smad3. J Biol Chem. 2003. 278: 43470-9.
394. Arroyo, A.G., et al. Differential requirements for alpha4 integrins during fetal and adult hematopoiesis. Cell. 1996. 85: 997-1008.
395. Hirsch, E., et al. Impaired migration but not differentiation of haematopoietic stem cells in the absence of beta1 integrins. Nature. 1996. 380: 171-5.
396. Liang, F., et al. The role of protein-tyrosine phosphatase 1B in integrin signaling. J Biol Chem. 2005. 280: 24857-63.
397. Cortes, J. and H. Kantarjian. New targeted approaches in chronic myeloid leukemia. J Clin Oncol. 2005. 23: 6316-24.
398. Taylor, G.P. and M. Matsuoka. Natural history of adult T-cell leukemia/lymphoma and approaches to therapy. Oncogene. 2005. 24: 6047-57.
399. Aversa, S.M., et al. Treatments of AIDS-related Kaposi's sarcoma. Crit Rev Oncol Hematol. 2005. 53: 253-65.
400. Hassan, R., et al. Acute myeloid leukemia following treatment with cladribine for hairy cell leukemia: a case report and review of the literature. Leuk Lymphoma. 2004. 45: 2149-52.
401. Cusnir, M. and Y.Z. Patt. Novel systemic therapy options for hepatocellular carcinoma. Cancer J. 2004. 10: 97-103.
402. Lawson, D.H. Choices in adjuvant therapy of melanoma. Cancer Control. 2005. 12: 236-41.
403. Shimizu, T., et al. A novel mechanism for imatinib mesylate (STI571) resistance in CML cell line KT-1: role of TC-PTP in modulating signals downstream from the BCR-ABL fusion protein. Exp Hematol. 2004. 32: 1057-63.
404. Zhang, J., et al. Roles of the SHP-1 tyrosine phosphatase in the negative regulation of cell signalling. Semin Immunol. 2000. 12: 361-78.
405. Coggeshall, K.M., et al. How do inhibitory phosphatases work? Mol Immunol. 2002. 39: 521-9.
406. Pavlick, K.P., et al. Role of reactive metabolites of oxygen and nitrogen in inflammatory bowel disease. Free Radic Biol Med. 2002. 33: 311-22.
407. Choi, I., et al. Interferon gamma delays apoptosis of mature erythroid progenitor cells in the absence of erythropoietin. Blood. 2000. 95: 3742-9.
408. Arias-Salgado, E.G., et al. PTP-1B is an essential positive regulator of platelet integrin signaling. J Cell Biol. 2005. 170: 837-45.

409. Fukada, T. and N.K. Tonks. Identification of YB-1 as a regulator of PTP1B expression: implications for regulation of insulin and cytokine signaling. Embo J. 2003. 22: 479-93.
410. Ahmad, F., et al. Improved sensitivity to insulin in obese subjects following weight loss is accompanied by reduced protein-tyrosine phosphatases in adipose tissue. Metabolism. 1997. 46: 1140-5.
411. Ahmad, F. and B.J. Goldstein. Increased abundance of specific skeletal muscle protein-tyrosine phosphatases in a genetic model of insulin-resistant obesity and diabetes mellitus. Metabolism. 1995. 44: 1175-84.
412. Dadke, S.S., et al. Elevated expression and activity of protein-tyrosine phosphatase 1B in skeletal muscle of insulin-resistant type II diabetic Goto-Kakizaki rats. Biochem Biophys Res Commun. 2000. 274: 583-9.
413. Wiener, J.R., et al. Overexpression of the protein tyrosine phosphatase PTP1B in human breast cancer: association with p185c-erbB-2 protein expression. J Natl Cancer Inst. 1994. 86: 372-8.
414. Tao, J., et al. Insulin stimulates tyrosine phosphorylation and inactivation of protein-tyrosine phosphatase 1B in vivo. J Biol Chem. 2001. 276: 29520-5.
415. Bukczynska, P., et al. The T-cell protein tyrosine phosphatase is phosphorylated on Ser-304 by cyclin-dependent protein kinases in mitosis. Biochem J. 2004. 380: 939-49.
416. Baines, M.G., et al. Decidual infiltration and activation of macrophages leads to early embryo loss. Am J Reprod Immunol. 1997. 37: 471-7.
417. Lee, H., et al. Identification of Phosphocaveolin-1 as a Novel Protein Tyrosine Phosphatase 1B Substrate. Biochemistry. 2006. 45: 234-240.