Tyrosine Phosphorylation Controls the Immune Response to Cancer

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Abstract

The protein tyrosine phosphatase PTP1B is a ubiquitously expressed enzyme that is involved in a variety of signalling pathways. Initially described as being a negative regulator of insulin receptor signalling, PTP1B garnered much attention as an anti-diabetic drug target. In recent years, investigations of PTP1B have uncovered both pro- and anti-oncogenic roles depending on the tissue and substrate in question. Tumor suppressive PTP1B functions have been described in esophageal cancer and lymphoma. Oncogenic functions, in contrast, have been characterized in breast, prostate, gastric, lung and colorectal cancers. The roles of PTP1B in cancer are still the subject of intense research, however several recent studies have begun to uncover its involvement in innate and adaptive immune signalling. Herein, we used various genetic approaches to understand the mechanism by which PTP1B influences the anti-cancer immunological response.

PTP1B and p53 double deficient mice rapidly succumb to B cell lymphomas with dramatic changes in B cell progenitor populations. To better understand the role PTP1B plays in B cell lymphomas, we challenged PTP1B null mice with a c-Myc driven mouse model of human Burkitt's lymphoma. We demonstrated that lymphoma challenged PTP1B deficient mice were more susceptible to rapid and aggressive lymphoma development. Infiltrating these lymphomas were large populations of immature dendritic cells (DCs) and other immune suppressive cell types. We discovered that in the absence of PTP1B, DCs are compromised in their antigen presentation capacity and are characteristic of an immature and tolerogenic state. We confirmed the impact of PTP1B on DCs activity by creating a tissue specific knockout mouse model, which when challenged with Myc-lymphoma behaved as the full body knockout mouse.

Extending our initial observations, we hypothesized that by modulating the activity of PTP1B we could enhance the expression of key DC maturation markers. Further investigation revealed that a dose-dependent mechanism exists within DCs where mice

heterozygous for PTP1B or the closely related TC-PTP promoted greater DC maturation and T cell activation. Notably these DCs were characterized by upregulation of MHC and costimulatory molecules, as well as increased IL-12 cytokine production. This finding led us to investigate the clinical utility of employing a PTP1B/TCPTP small molecule inhibitor towards developing a DC based immunotherapy. Treatments of DCs from these mouse models as well as those derived from human pancreatic cancer patients resulted in increased expression of DC maturation markers. The inhibitor-treated DCs are more potent antigen presenting cells, capable of eliciting a higher level of antigen-specific T cell activation and induce superior tumor clearance. We found that enhanced DC activity is achieved through increased production of IL-12, IFN-γ and downstream STAT1 and STAT4 activation. These pathways are known to be essential in the activation of DC function and the induction of anti-tumor Th1-polarized immunity. In conclusion, we have shown that PTP1B/TC-PTP inhibitors represent a novel immunotherapeutic approach, which has the potential to reduce cancer development.

Résumé

La protéine tyrosine phosphatase PTP1B est une enzyme ubiquitaire impliquée dans une grande variété de voies de signalisation. Initialement décrite comme un régulateur négatif du récepteur à l'insuline, PTP1B attire beaucoup l'attention en tant que cible thérapeutique pour le traitement du diabète. Ces dernières années, les études décrivent PTP1B comme proto- et anti-oncogénique dépendamment du tissue où elle est exprimée. Dans le cancer de l'œsophage et les lymphomes, PTP1B agit en tant que suppresseur de tumeur. En contraste, PTP1B a une fonction oncogénique dans les cancers du sein, de la prostate, du poumon, de l'estomac et du colon. Le rôle de PTP1B dans le cancer est toujours un sujet de recherche d'actualité, par contre de plus en plus d'études ont répertoriées son implication dans les réponses immunitaires innée et acquise. Ici, nous utilisons plusieurs approches génétiques pour comprendre le ou les mécanismes par lequel(s) PTP1B influence la réponse immunitaire anti-tumorale.

Les souris double-déficientes en PTP1B et p53 succombent rapidement à un lymphome de type B comme l'indiquent les changements dramatiques observés dans la population de cellules B progénitrices. Afin de mieux comprendre le rôle de PTP1B dans le lymphome de type B, nous avons utilisé la souris transgénique E-mu-myc, un modèle du lymphome de Burkitt chez l'humain. Nous avons démontré que les souris déficientes en PTP1B injectées avec les cellules tumorales sont plus sensibles au développement rapide et agressif d'un lymphome. Ces lymphomes sont constitués d'une importante population de cellules dendritiques (DCs) immatures ainsi que d'autres types de cellules immunosuppressives. Nous avons constaté qu'en absence de PTP1B, les DCs sont caractérisées par une capacité de présentation des antigènes compromise et par un état immature et tolerogénique. De plus, nous avons généré une souris knock-out pour PTP1B tissu spécifique dans les cellules dendritiques qui une fois injectées avec les des cellules tumorales de lymphome agit comme le knock-out total, ce qui confirme l'impact de PTP1B sur l'activité des DCs.

Un examen plus approfondi a révélé qu'il existe un mécanisme dose-dépendent où les souris hétérozygotes pour PTP1B ou TC-PTP promeuvent la maturation et l'activation des DCs. Notamment, ces DCs sont caractérisées par une augmentation de la régulation des MHC et molécules co-stimulées ainsi qu'une augmentation de la production de la cytokine IL-12. Grâce à ces observations, nous nous sommes penchés sur l'utilité clinique d'employer des inhibiteurs de PTP1B/TC-PTP dans le développement d'une immunothérapie à base de DCs. Les traitements avec inhibiteurs de DCs provenant des souris mentionnées ci-haut et de DCs dérivées de patients souffrant du cancer du pancréas montrent qu'elles sont préférentiellement activées. Les DCs traitées avec des inhibiteurs montrent une efficacité accrue à présenter leurs antigènes, suscitent une activation plus élevée de l'antigène spécifique des lymphocytes T et induisent une meilleure clairance des tumeurs. Nous avons déterminé que l'activité élevée des DCs implique une augmentation de la production de IL-12, IFN-g et des protéines STAT1 et STAT4. Ces voies de signalisation sont essentielles à l'activation de la fonction des DCs et à l'induction de la réponse immune polarisée Th1 anti- tumorale. En conclusion, nous avons montré que le traitement des cellules dendritiques avec des inhibiteurs de PTP1B/TC-PTP représente une nouvelle approche immunothérapeutique avec le potentiel de réduire la progression du cancer.

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PREFACE

This is a manuscript-based thesis, which consists of one published review article, one research article in submission, and two research articles currently in preparation for which I will be first and co-first author.

Chapter 2: Feldhammer M, Uetani N, Miranda-Saavedra D, Tremblay ML. (2013). PTP1B a simple enzyme for a complex world. Crit Rev Biochem Mol Biol **48**(5): 430-45

Chapter 3: Matthew Feldhammer*, Claudia Penafuerte*, John R. Mills, Eva Mignon, Ailsa Lee Loy, Noriko Uetani, Gerard Karsenty, Jerry Pelletier and Michel L. Tremblay (2015). PTP1B deficiency leads to enhanced lymphoma progression due to compromised dendritic cell function. *Manuscript submitted*

Chapter 4: Claudia Penafuerte*, Matthew Feldhammer*, George Zogopoulos and Michel L. Tremblay (2015). Targeting tyrosine phosphatases as the basis for a novel dendritic cell based immunotherapy. *Manuscript in preparation*

Chapter 5: Matthew Feldhammer, Claudia Penafuerte, Nikita Desai, Valerie Vinette, and Michel L. Tremblay (2015). STAT1 and STAT4 modulate the preferential maturation of dendritic cells by the protein tyrosine phosphatases, PTP1B and TC-PTP. *Manuscript in preparation*

*Co-first author

CONTRIBUTON OF AUTHORS

Chapter 3:

I performed all the experiments except: Tail vein injections of tumor cells were performed by Ailsa Lee Loy and Dr. Noriko Uetani under my direct supervision. Dr. John Mills provided the lymphoma cells. The immune infiltration experiments (Figure 3.3) were performed by Dr. Claudia Penafuerte and myself. FACS staining for the immune infiltration and phenotypic analysis of BMDCs was performed by Dr. Claudia Penafuerte and I conducted the data analysis. T cell activation experiments (Figures 3.5 E-F) were performed by Dr. Claudia Penafuerte. The work in chapter 3 and all subsequent chapters was completed under the supervision of Dr. Michel L. Tremblay.

Chapter 4:

I performed all experiments except: Phenotypic analysis of BMDCs (Figure 4.1 and figure 4.7) was performed by both myself and Dr. Claudia Penafuerte. T cell activation experiments were conducted by Dr. Claudia Penafuerte (Figure 4.2 C-D). All of the mouse tumor studies were conducted by both Dr. Claudia Penafuerte and myself. I generated the stable luciferase expressing cells lines and assisted in the tumor implantations and therapeutic DC treatments. Dr. Claudia Penafuerte monitored the mice by caliper measurements and I conducted the in vivo imaging experiments. The PTP1B inhibitor specificity assay was performed by Isabelle Aubry. Human samples were provided by Dr. George Zogopoulos. Dr. Claudia Penafuerte performed the ELISpot experiments.

Chapter 5:

I performed all experiments except: Generation of BMDCs was performed by both Dr. Claudia Penafuerte and myself. Microarrays experiments were performed at Genome Quebec and analyzed by Nikita Desai. qRT-PCR experiments were performed by Valerie Vinette and myself.

ORIGINAL CONTRIBUTION TO KNOWLEDGE

Chapter 3: PTP1B deficiency leads to enhanced lymphoma progression due to compromised dendritic cell function

- Loss of PTP1B accelerates myc driven lymphomagenesis and decreases overall survival.
- Tumour infiltrates in PTP1B deficient mice display increased numbers of immature and tolerogenic DCs as well as other immune suppressive cell types.
- PTP1B deficient DCs are phenotypically immature and deficient in their ability to activate $CD4^+$ T cells.
- PTP1B deficient DCs display hyperphosphorylation of STAT3 and STAT5.
- Mice with a DC specific deletion of PTP1B display the same survival defects as the full body KOs upon lymphoma tumor challenge.

Chapter 4: Targeting tyrosine phosphatases as the basis for a novel dendritic cell based immunotherapy

- Single copy deletions of PTP1B in DCs increase the expression of key maturation markers and the ability to activate $CD8^+$ T cells.
- Therapeutic vaccination of tumor bearing mice with PTP1B heterozygous DCs significantly delays tumor growth.
- Treatment of DCs with a PTP inhibitor increases the expression of key DC maturation markers.
- Therapeutic vaccination of tumor bearing mice with PTP inhibitor-treated DCs significantly delays tumor growth.
- Treatment of pancreatic cancer patient DCs with a PTP inhibitor increases the activation of tumor antigen-specific T cells.
- Developed a novel DC immunotherapy pipeline based on the use of PTP inhibitors.

Chapter 5: STAT1 and STAT4 modulate the preferential maturation of dendritic cells by the protein tyrosine phosphatases, PTP1B and TC-PTP

- Modulation of PTP1B and TC-PTP activity in DCs results in hyperphosphorylation of STAT1, STAT4 and Akt.
- Inhibitor treated DCs upregulate key genes associated with DC maturation as well as genes associated with positive cellular development, differentiation and immune processes.
- Successfully generated a DC tissue specific double heterozygous mouse model of PTP1B and TC-PTP.
- Inhibitor treated and double heterozygous DCs secrete increased amounts of the key Th1 cytokines IL-12 and IFN-γ.

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
AgRP	Agouti-Related Peptide
BCR	B Cell Receptor
BMDC	Bone Marrow-Derived Dendritic Cell
BP	Blood Pressure
CTL	Cytotoxic T Lymphocyte
CTLA4	Cytotoxic T-Lymphocyte-Associated Protein 4
DC	Dendritic cell
DLBCL	Diffuse Large B-Cell Lymphoma
eIF2α	Eukaryotic Translation Initiation Factor 2α
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-Linked ImmunoSpot
ER	Endoplasmic Reticulum
FACS	Fluorescence Activated Cell Sorting
FDA	Food and Drug Administration
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GWAS	Genome-Wide Associate Study
iDC	Immature Dendritic Cell
IgH	Immunoglobulin Heavy Chain
IgL	Immunoglobulin Light Chain
IKB	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer Inhibitor
IKK	I Kappa B Kinase Complex
IL	Interleukin
IFN	Interferon
IR	Insulin Receptor

IRE1	Inositol-Requiring Enzyme
JNK	C-Jun N-Terminal Kinase
KO	Knockout
LC	Langerhans Cell
LPS	Lipopolysaccharide
mDC	Myeloid Dendritic Cell
MDSC	Myeloid-Derived Suppressor Cell
moDC	Monocyte-Derived Dendritic Cell
MHC	Major Histocompatibility Complex
MSCV	Murine Stem Cell Virus
MyD88	Myeloid Differentiation Primary Response Gene 88
NDR	NOD-like receptors
NF-κB	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer
NK	Natural Killer cell
NKT	Natural Killer T cell
NLR	NOD-Like Receptor
NLS	Nuclear Localization Signal
OVA	Ovalbumin
PaC	Pancreatic Cancer
PD1	Programmed Cell Death Protein 1
pDC	Plasmacytoid Dendritic Cell
POMC	Pro-opiomelanocortin
PERK	Protein Kinase R-Like Endoplasmic Reticulum Kinase
PGE2	Prostaglandin E2
PTK	Protein Tyrosine Kinase
PTM	Post Translational Modification
PTP	Protein Tyrosine Phosphatase
PTPN1	Protein Tyrosine Phosphatase Non-Receptor Type 1
PAMP	Pathogen Associated Molecular Pattern

PRR	Pattern Recognition Receptor
ROS	Reactive Oxygen Species
SDF-1	Stromal Cell-Derived Factor 1
SNP	Single Nucleotide Polymophism
STAT	Signal Transducers and Activators of Transcription
T2D	Type 2 Diabetes
TAA	Tumor-Associated Antigen
TC-PTP	T-Cell Protein Tyrosine Phosphatase
TGF-β	Transforming Growth Factor Beta
Th	T Helper Cell
TNF	Tumour Necrosis Factor
TLR	Toll-like Receptor
Treg	Regulatory T cell
UPR	Unfolded Protein Response
VEGF	Vascular Endothelial Growth Factor
WT	Wild-Type

CHAPTER 1: INTRODUCTION

1.1 General introduction

The immune system is complex network of cells, organs and tissues, which work together to protect the body from pathogens. The human body provides an ideal location for the growth and reproduction of many bacteria, parasites and viruses. Our immune system therefore is primarily tasked with preventing and or limiting the extent to which these pathogens can infect or invade us in order to maintain our health. The immune system's main protective mechanisms consist of two tightly regulated and non-mutually exclusive responses. The cell mediated or innate immune response is the first barrier to foreign invaders. This response is rapid and capable of directing immune components to the site of infection without the production of antibodies. The adaptive arm of the immune system is highly specialized to respond to specific threats through the production of antibodies and antibody mediated responses, which are capable of recognizing an almost infinite number of protein antigens. These responses are slower than the cellular mediated immune response but they are highly specific and confer long lasting immunity.

Both the innate and the adaptive arms are tightly regulated being governed by both cellular and chemical cues. The balance between immune activation and immune suppression is pivotal in maintaining health. An unrestricted immune response can have disastrous effects on the body, including chronic inflammation, autoimmune disorders and cancer. Recently, an extensive amount of research has been aimed at understanding links between cancer and immunity. The immune system is capable of identifying tumors and mounting a response against them. However, this response is usually insufficient to achieve tumor clearance and the cancer will continue to proliferate and metastasize resulting in host mortality. The goal then of understanding the relationship between the immune system and cancer has driven the development of potent and specific immunotherapies. These therapies are geared to either enhance the immune response towards a specific tumor antigen or suppress the ability of the tumor itself to proliferate. Several of these therapies are currently available and are

increasingly becoming the standard of care in many cancer types owing to their ability to provide significant improvements in both quality and length of life, without the cytotoxic side effects of chemotherapies.

Several types of immunotherapy exist, but all share the common concept of improving upon the body's own defenses to treat the disease. One approach aims to increase tumor recognition by the immune system by either activating or restoring the anti-tumour immune response. Dendritic cells (DCs) can be used to program immune effector cells to recognize and eliminate the tumor. Dendritic cell-based immunotherapy is currently approved for the treatment of metastatic prostate cancer (Provenge®) and is being actively investigated in clinical trials for applications in other malignancies. However, one of the main obstacles in this approach is the immune suppressive nature of the tumor microenvironment. Dendritic cell maturation and activation can be hindered by cues from suppressive cytokines. Therefore, understanding such processes in DCs is critical in the development of the next generation of cancer vaccines. We are now beginning to understand these complex signalling networks and are developing ways to exploit them in order to enhance the efficacy of future cancer therapies. Regulation of such networks is largely reliant on post-translational modifications (PTMs) of signalling molecules. One of the most common PTMs is tyrosine phosphorylation, a process that is controlled by two opposing groups of enzymes, the protein tyrosine kinases (PTKs) and the protein tyrosine phosphatases (PTPs). PTK and PTP signalling plays an important role in regulating all manner of cellular processes and dysregulation of either enzyme can result in a wide range of consequences including altered immune cell function and cancer.

1.2 The immune system

The immune system is primarily tasked with protecting the body from infection. Bacteria, viruses, fungi and parasites attempt to infiltrate the body and the immune system is responsible for recognizing these foreign pathogens and responding

accordingly. The resulting response is accomplished by immune cells, which originate in the bone marrow and migrate to the periphery and undergo differentiation and activation in their highly specialized immune compartments. When a microorganism manages to infiltrate the body it first comes into contact with the innate immune system. The innate immune system is comprised of the physical epithelial barrier as well as phagocytic macrophages, DCs natural killer (NK) cells, granulocytes, and mast cells, and generally results in the release of inflammatory cytokines which can activate the complement system in a first attempt to clear the pathogen [1]. The adaptive immune response, though slower to react upon infection, is able to mount a highly specific and sustained response to a target antigen. This branch of the immune system is highly relevant to the anti-cancer immune response. Adaptive immunity is primarily achieved through the activation and recruitment of antigen presenting cells (APCs) as well as a variety B and T lymphocytes, which work to produce antibodies against the foreign antigen.

The task of recruiting and activating cells of both arms of the immune response is tightly regulated through cytokine signalling. Cytokines are comprised of interleukins (ILs), interferons (IFNs) and hematopoietic growth factors, and are the main messengers in the immune system. Cytokines play a variety of roles including activating or suppressing the differentiation and maturation of certain cell types as well as modulating the balance between the innate and adaptive immune response [2]. The innate and adaptive immune responses are not mutually exclusive processes and often work together in order to best achieve immunity. This is particularly relevant in a cancer context where immune suppression, macrophage recruitment and inflammation as well as secretion of inhibitory cytokines and downregulation of antigenic factors are often employed by tumors to promote their growth and evade host immune surveillance [3, 4]. At the intersection of the innate and adaptive immune systems are specialized APCs known as DCs. DCs are capable of sampling the environment, capturing invading pathogens or tumor antigens and inducing either tolerance or immunity depending on

the antigen and cytokine environment present. Therefore, DCs are crucial determinants for tumor surveillance and the induction of anticancer immunity [5].

1.2.1 Innate immunity

Primarily a nonspecific defense mechanism, cells of the innate immune system can detect pathogen associated molecular patterns (PAMPs) in order to identify and respond to foreign pathogens. The detection of these PAMPs is carried out via non-clonal recognition receptors such as toll like receptors (TLRs), NOD-like receptors (NLRs), helicases as well as lectins [6]. TLRs are preferentially expressed on the surface of cells in the innate immune system including DCs, NK cells and macrophages which allows them to act as immune sentinels capable of sensing the environment [7]. Binding of PAMPs onto TLRs triggers upregulation and release of pro-inflammatory cytokines resulting in macrophage and NK cell recruitment in order to clear the foreign pathogen. Currently 10 and 12 TLRs have been identified in human and mouse respectively. These pattern recognition receptors (PRRs) are capable of recognizing a wide range of PAMPs such as bacterial lipopolysaccharide (LPS), peptidoglycan, single and double stranded DNA [8].

Many TLRs can activate overlapping intracellular signalling pathways resulting in the production of inflammatory cytokines IL-1, IL-6, tumor necrosis factor alpha (TNFα) and type I interferon (IFN-γ). In DCs, bacterial LPS will bind to and initiate TLR4 signalling. This results in the adaptor protein myeloid differentiation primary response gene 88 (MyD88) being recruited to the TLR4 receptor. MyD88 is a common adaptor protein and shares overlapping roles in many TLR signalling pathways. MyD88 will activate the inhibitor of nuclear factor kappa-B kinase (IKK) complex, which will phosphorylate the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα), the negative repressor of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [8]. NF-κB is usually held in an inactive state in the cytoplasm due to IκBα masking the nuclear localization signal (NLS) of the p65 subunit [9]. Once the IKK complex phosphorylates IκBα, it dissociates from NF-κB allowing it to enter the nucleus

and bind to various gene elements promoting the transcription of a variety of pro-inflammatory gene sets, including IFN-γ and IL-12 [10].

1.2.2 Adaptive immunity

In contrast to innate immunity, adaptive immunity refers to the induction of antigen-specific lymphocytes, and the development of immunological memory [1]. The primary lymphocytes that mediate this task are the antibody producing B cells and $CD4^+$ and $CD8^+$ T cells. Initiation of the adaptive immune response first requires an APC such as a DC to encounter a foreign pathogen. Upon PRR recognition, the pathogen is engulfed, degraded, and the derived antigens are presented on its cell surface to activate pathogen specific lymphocytes. In addition to lymphocyte activation, DCs are capable of secreting cytokines that can modify resulting innate and adaptive immune responses. B and T lymphocyte subsets are located in peripheral organs of the immune system and can undergo rapid differentiation and activation upon antigen and cytokine exposure.

B and T lymphocyte precursors are generated in the bone marrow. However, T cell maturation occurs in the thymus as opposed to B cells which mature in the bone marrow and the spleen [11]. In order to generate protective immunity against a variety of pathogens, the immune response is tightly coordinated by various T cell subsets including T helper (Th) 1, Th2, and Th17 effector $CD4^+$ T cells. The differentiation of naïve $CD4^+$ T cells into these subsets is highly reliant upon the antigen and the cytokine environment they encounter [12]. Th1 polarized T cells are primarily characterized by high amounts of IFN-γ, IL-2 and TNF-β production. These cells are particularly adept at macrophage activation, eliciting cell-mediated cytotoxicity and enhancing the pro-inflammatory response [13]. These responses are highly beneficial for killing intracellular parasites as well as tumors. Th2 polarized T cells produce large amounts of IL-4, IL-5, IL-6, IL-9, IL-10, and IL- 13, and primarily evoke an IL-10 mediated anti-inflammatory response, counteracting several functions of macrophages providing a phagocyte-independent protective response [14]. Thus an effective immune strategy requires a

balance be achieved between Th1 and Th2 responses tailored to the individual requirements of the pathogen. In addition, a successful immune response requires the ability for negative regulation in order to limit tissue damage due to inflammation. Several mechanisms exist in order inhibit immune responses including secretion of various cytokines such as IL-10 as well as the production of T regulatory cells (Tregs) as the primary cellular immune suppressors [15]. Tregs are recruited by tumor cells in order to counteract anti-tumor immunity, a subject which will be discussed in more detail later on [16].

$CD8^+$ T cells are distinguished from the $CD4^+$ subset by the presence of the CD8 surface glycoprotein and absence of CD4 [17]. Resting naïve $CD8^+$ T cells are able to react to a wide variety of pathogens by rapid expansion and differentiation into cytotoxic effector cells. This cascade is initiated by an APC, such as a DC, which will trigger $CD8^+$ T cell expansion and activation towards a target antigen. Once they have encountered their target antigen, activated, antigen-specific $CD8^+$ T cells will secrete the cytotoxins perforin and granzyme, triggering apoptosis in the target cell [18].

B lineage cells are the primary mediators of the humoral or antibody mediated immune response. B cells provide specific and long lasting protection from a diverse set of pathogens. This is accomplished primarily through antibody secretion by plasma B cells, which are differentiated B effector cells capable of secreting large amounts of pathogen-specific antibodies to neutralize target pathogens [19]. Plasma B cells exist as clonal populations, each clone capable of recognizing and producing antibody in response to a particular antigen. This specificity is determined by the nature of the B cell receptor (BCR) on the cell surface. BCR specificity is determined early in B cell development through the error prone combinatorial VDJ rearrangement. In brief, the process involves the re-arrangement of the variable (V), diversity (D) and joining (J) gene segments in the immunoglobulin heavy chain (IgH) locus and the V and J gene segments on the immunoglobulin light chain (IgL) locus [20]. B cell activation involves several signals but

primarily requires antigen recognition by the BCR resulting in B cell proliferation and differentiation. Activated B cells can differentiate into either plasma B cells or memory B cells. Memory B cells provide the long lasting immune protection even upon antigen re-exposure. While B cells are capable of recognizing and responding to soluble antigens, the major form of antigen that activates B cells is that presented on the surface of helper T Cells [21] [22].

1.2.3 Dendritic cells

For his work in the identification of DCs and characterizing the role they play in the adaptive immune response, the late Ralph Steinman was awarded the 2011 Nobel Prize in Physiology or Medicine. Dendritic cells are the most potent APCs of the immune system and are therefore often referred to as professional APCs. Synthesized as monocytic precursors in the bone marrow, immature DCs survey the immune environment in peripheral tissues and the blood with a high capacity to uptake and process antigen. Upon antigen exposure DCs migrate into the draining lymph nodes where they present antigen to T-lymphocytes, thus stimulating their activation [23]. Dendritic cells therefore form the critical interface between the innate and adaptive immune response due their ability to utilize PRRs such as TLRs to recognize a variety of pathogens and subsequently activate antigen-specific T cell responses [24]. Activated DCs present antigen to either $CD8^+$ or $CD4^+$ T cells in the context of major histocompatibility complex (MHC) class I or class II molecules, respectively. Antigen presentation also requires the interaction of costimulatory molecules which when absent can induce immune tolerance [25]. In addition, activated DCs produce a variety of cytokines that drive T cell priming as well as effector differentiation. DCs have the uncommon ability to load extracellular derived antigens onto MHC class I molecules and prime $CD8^+$ T cells in a process known as cross-presentation. This process is essential for the initiation of immunity against extracellular pathogens that do not invade APCs such as viruses and tumors [26]. For these reasons the therapeutic potential of DCs is actively

being investigated in academia as well as private industry for use in therapies against cancer and auto-immunity [5].

1.2.3.1 DC subsets

Although first observed by Paul Langerhans in the late 19th century, it was not until the work of Ralph Steinman and Zanvil Cohn that DCs garnered the interests of the scientific community at large. Observed originally in the spleen, Steinman described this rare cell population for which he coined the term DC as having a characteristic stellate morphology [27]. Since this initial description it has become clear that DCs constitute a vital component in the immune system. Several DC subsets exist in both humans and mice that differ in their phenotypic and functional properties. A list of DC subsets and their secreted cytokines and locations is included in table 1 (adapted from [24]). The two main DC subsets are the 'classical' or myeloid DCs (mDCs) and the plasmacytoid DCs (pDC). Despite many differences, the majority of DC subsets express high amounts of MHC class II and the integrin CD11c on their surface (human pDCs being the exception).

mDCs can be found in the peripheral tissue, secondary lymphoid organs as well as the blood. Upon activation, antigen-loaded mDCs enter the draining lymph nodes through afferent lymphatics where they encounter and activate T lymphocytes [28]. mDCs can be further sub-divided based on their location. In the skin there exists three subsets. Located within the epidermis there are the Langerhans cells (LCs), which are highly potent activators of $CD8^+$ T cells [29]. Located in the dermis there are $CD1a^+$ DCs and $CD14^+$ DCs [30]. $CD14^+$ DCs in contrast to LCs are more potent inducers of the antibody-mediated immune response [31]. $CD1a^+$ DCs are reported to share some common functions with both $CD14^+$ DCs and LCs although they are less efficient in the activation

DC subset	Cytokines produced	Antigen presentation capacities	Distribution
Plasmacytoid DC	Type 1 IFN	Present and cross-present peptides	Lymphoid organs
CD8α+ cDC	IL-12	Cross-presentation, MHC I to CD8+ T cells	Lymphoid organs
CD103+ cDC	IL-1β and IL-6	Cross-presentation, MHC I to CD8+ T cells	Peripheral tissues
CD11b+ cDC	IL-6 and IL-23	MHC class II to CD4+ T cells	Lungs and gut
Interstitial (dermal) DC	TNF and IL-12	MHC class II to CD4+ T cells	Peripheral tissues
Langerhans cell	IL-15	Cross-presentation, MHC I to CD8+ T cells	Epidermis
Monocyte-derived DC	TNF, IL-12 and type I IFN	Present and cross-present peptides	Lymph nodes and tissues

Table 1. List of common DC subsets, cytokines produced, antigen presentation capacities and their distributions, adapted from [24] and [32-36]

of $CD8^+$ T cells than LCs [29]. The difference in function between $CD14^+$ DCs and LCs is likely due to the cytokines each secretes. LCs and $CD1a^+$ DCs produce the $CD8^+$ T cell response enhancing cytokine IL-15. In contrast $CD14^+$ DCs produce a wider variety of cytokines including IL-1β, IL-6, IL-8, IL-10, IL-12, granulocyte–macrophage colony stimulating factor (GM-CSF), as well as transforming growth factor (TGF)-β, which contribute to their ability to program B and T lymphocytes into enhancing antibody production [37].

Plasmacytoid DCs reside in the blood and in contrast to mDCs enter the lymph nodes through endothelial venules as opposed to the afferent lymphatics [38]. In humans, pDCs unlike most other DC subsets express undetectable levels of the surface marker CD11c and low levels of MHC class II. Plasmacytoid DCs are highly adept at recognizing viral particles through expression of TLRs -7 and -9 on their surface which recognize single-stranded RNA and CpG DNA, respectively [39]. The hallmark of pDCs compared to other DC subsets is their ability to secrete large amounts of type I IFN [38, 40]. Plasmacytoid DCs are thus able to elicit the recruitment and activation of a variety of immune cell types including NK cells [41] and plasma cells [42].

Despite a large number of subsets, DCs represent a very small fraction of the total number of immune cells present in the body. Plasmacytoid DCs for example only constitute 0.3-0.5% of human peripheral blood [43]. This scarcity limited the ability of scientists to study DCs. To address this issue, Dr. Steinman's lab developed a protocol for the generation of DCs from mouse bone marrow (BMDCs) [44]. Using the cytokines GM-CSF and IL-4, monocyte bone marrow precursors will differentiate into immature DCs over the course of 6 days. BM-DCs thus represent a useful tool for scientists to study DC biology.

1.2.3.2 DC maturation

Dendritic cells exist in two basic functional states; immature DCs (iDCs) have a high phagocytic capacity and can present self-antigens to T cells, resulting in immune tolerance through T cell deletion or the activation of Tregs [6]. Mature (activated) DCs in contrast have a very low capacity for antigen uptake but can present antigen to T lymphocytes, inducing their differentiation into effector cells [45]. Interestingly, immature DCs have been found in large numbers in cancer patients and likely contribute to localized immune suppression at the tumor site [46]. Mature DCs on the other hand are actively being investigated for their utility in generating anti-cancer vaccines and one DC based immunotherapy formulation has already been approved for use in metastatic prostate cancer patients [47].

Considering the tolerogenic nature of iDCs in light of the vast therapeutic potential of DC-directed antigen-specific T cell responses, a proper understanding of the pathways involved in DC activation is essential. DC activation is a complex process involving a number of inter- and intracellular signals, resulting in dramatic phenotypic changes. Activated DCs are characterized by a high expression of surface MHC class II and costimulatory molecules CD80, CD86 and CD40 as well as secretion of large amounts of IL-12 [48] [49]. DC activation is initiated by antigen binding onto TLRs, which enhances peptide loading onto MHC molecules [50]. For example, LPS binds to TLR4, which is coupled to the adaptor molecule MyD88 and leads to activation of NF-κB through a series of kinase-regulated phosphorylation reactions. The NF-κB transcription factor is critical for driving gene expression required for DC activation, as inhibition of NF-κB activation was shown to block DC maturation [51]. One of the myriad of transcriptional targets that NF-κB binds is the IL-12 promoter [52]. DC secretion of large amounts of IL-12 is one of the hallmarks of DC activation and drives naive T cells towards a pro-inflammatory Th1 cell fate [53]. DCs also express the IL-12 receptor on their surface and are therefore able to drive their own activation in an autocrine fashion [10]. Robust expression of MHC and costimulatory molecules on the cell surface coupled with

secretion of cytokines by mature DCs is paramount because they directly define the T cell priming ability of the cell [45].

1.2.3.3 DC-mediated T cell activation

DCs are not effector cells themselves and therefore require an interaction with T lymphocytes in order to coordinate the adaptive immune response. Depending on the nature of this interaction naive $CD4^+$ or $CD8^+$ T cells can differentiate into a number of different effector cells. Naive $CD4^+$ T cells can differentiate into Th1, Th2, Tregs, T follicular helper cells, which help B cells to differentiate into plasma cells. In addition naive $CD4^+$ T cells can also differentiate into Th17 cells (Th17 cells are primarily defined by secretion of IL-17 and play important roles in inflammation and autoimmunity primarily mediated through the recruitment of neutrophils and macrophages [54]) [6]. In contrast to the large number of effector cells arising from $CD4^+$ T cells, naive $CD8^+$ T cells give rise to cytotoxic T lymphocytes (CTLs), which are critical effectors for the induction of antitumor immunity [55, 56]. Dendritic cells are also capable of interacting with B cells both directly and indirectly through the expansion of $CD4^+$ T helper cells [57, 58]. DC activation of T cells therefore requires three key signals: 1) Antigen presentation in the context of MHC class I or class II molecules, 2) Interaction of costimulatory molecules such as CD40, CD80 or CD86, and 3) Cytokine stimulation provided by DCs [59].

Mature DCs present antigen to naive $CD4^+$ T cells in the context of MHC class II molecules leading to the differentiation of a number of different effector cell types. The presence of various cytokines and costimulatory molecules is the primary determinant in the effector cell fate [60]. DCs can differentiate naive $CD4^+$ T cells into Th1 effectors based on the production of IFN-γ and IL-12, resulting in the activation of transcription factors, signal transducers and activators of transcription (STAT) 1 and STAT4 [61]. In contrast, the production of IL-4 leads to the activation of STAT6 and polarizes naive $CD4^+$

T cells towards a Th2 response [62]. DCs are also capable of inducing the differentiation of immune suppressive Tregs through the production of IL-2 [63].

The communication between DCs and T cells however is not a simple one-way street, as Tregs were shown to suppress DC maturation by directly downregulating the expression of costimulatory molecules CD80 and CD86 [64, 65]. In addition, the topic of DC 'licensing', whereby the activation of $CD8^+$ CTLs requires that DCs must first encounter antigen-specific T helper cells remains the topic of much research [66, 67]. $CD8^+$ T cells and DCs both express the costimulatory molecule CD40, and $CD4^+$ T cells express the CD40-ligand (CD40-L). CD40 stimulation encourages DCs to produce the pro-inflammatory Th1 cytokines, IL-12 and IFN-γ as well as to upregulate antigen presentation and costimulatory molecule expression, resulting in an enhanced ability to recruit and prime CTLs [68, 69]. Therefore, the diversity of DC-based effector responses is highly dependent upon the cytokine environment within which the interactions occur.

1.2.3.4 Tolerogenic DCs

In stark contrast to their roles in coordinating both innate and adaptive immunity in response to infection, it is well accepted that DCs are also capable of inducing T cell tolerance [70]. Tolerogenic DCs play a critical role in the induction of self-tolerance, thymic DCs contribute to negative selection of self-reactive T cell precursors, while peripheral DCs maintain immune homeostasis and regulate autoimmunity [71]. As previously defined, DCs can exist in both a mature and immature state and the potential of DCs to induce tolerogenic responses is directly related to their maturation status [72]. DCs can induce both central and peripheral tolerance via a number of mechanisms including: the induction of T cell apoptosis, induction of T cell anergy and the differentiation of Tregs [73]. iDCs presenting low levels of self-peptide-MHC complexes and limited costimulatory molecule expression provide insufficient signal 1 or signal 2 molecules and result in T cell anergy or apoptosis [70].

Mature DCs have also been shown to induce tolerance and T cell anergy, especially when exposed to anti-inflammatory cytokines IL-10, TGF-β, vascular endothelial growth factor (VEGF) and prostaglandin E2 (PGE2) in the tumor microenvironment [74-76]. Mature tolerogenic DCs in the tumor microenvironment express low levels of MHC class I, class II as well as costimulatory molecules and produce high amounts of IL-10 which blocks DC maturation, leading to T cell anergy and induction of Tregs [77-79]. The result of tolerogenic DCs in the tumor microenvironment further promotes an immune suppressive state, which permits the tumors to evade the host immune response [59, 80, 81].

1.2.3.5 JAK-STAT signalling in DCs

A number of cytokines involved in DC activation transduce their signals via the Janus activated kinase (JAK) family of tyrosine kinases and STAT transcription factors. JAK-STATs are crucial components of diverse signal-transduction pathways that are actively involved in the regulation of a variety of cellular functions (a more general and mechanistic explanation of JAK-STAT signalling is available in **chapter 2**.) DC maturation is directly influenced by the activation state of several STAT transcription factors and their subsequent gene expression profiles. STAT1 [82] and STAT4 [83] have been shown to be essential for DC maturation whereas STAT3 plays an inhibitory role [84].

As previously mentioned, IL-12 secretion by DCs is a hallmark of maturation. IL-12 induces tyrosine phosphorylation and activation of the IL-12R through the activation of JAK2 and TYK2, which, in turn, phosphorylate and activate STAT4 [85, 86]. This pathway is essential for DC activation in an autocrine fashion, which leads to an enhanced ability of DCs to polarize T cells towards a Th1 response [87]. STAT1 activation downstream of JAK1 is essential for DC activation and was shown to be required for driving expression of the key costimulatory molecule CD40, as well as the surface integrin and pan DC marker CD11c [82]. Experiments using STAT1-deficient mice demonstrated the requirement of STAT1 in DCS for the production of IL-12 via IFN-γ-initiated signalling

[88]. In contrast to the positive roles STAT1 and STAT4 play in driving DC maturation, STAT3 has an opposing function. STAT3 is perhaps the most well characterized of all the STAT molecules as phosphorylation of STAT3 downstream of JAK2 and Tyk2 has been shown to play powerful roles in the promotion of tumor cell proliferation, survival, invasion and immunosuppression [89]. Using IL-10-deficient mice, Hoentjen et al. demonstrated that activated STAT3 blocks NF-κB recruitment to the IL-12p40 promoter, thereby inhibiting the expression of IL-12p40 mRNA [52]. Around the same time of the previous study further evidence was put forth to confirm the inhibitory activity of STAT3 in DCs. A paramount study by Kitamura et al. demonstrated that activation of the IL-6-STAT3 signalling pathway in DCs was able to suppress MHC class II expression and effectively abrogate the $CD4^+$ T cell response [90]. Several more recent studies have shown that inhibiting STAT3 activity in DCs using RNA interference enhances DC cytokine secretion and subsequent T cell activation [91, 92]. These studies highlight the importance of tyrosine phosphorylation in the regulation of proper DC maturation and function.

1.2.4 Immunotherapy

Immunotherapy is the utilization of the bodies own intrinsic defense mechanisms to fight cancer. This approach to cancer treatment represents the next generation of therapeutic options being currently investigated and utilized to enhance patient outcomes. Until recently cancer treatment was restricted to broad-spectrum chemotherapy and radiation therapy regimens which for a large part were, not tumor specific, highly cytotoxic and induced a litany of adverse side effects [93]. The notion of using the body's own immune system in the fight against cancer is not a new concept. As far back as the 18^{th} century it was observed that certain cancer patients with concomitant bacterial infections would undergo remission [94]. It was not for another 100 years however that in the late 19^{th} century that a New York surgeon by the name of William Coley devised a study to investigate this phenomenon. Dr. Coley injected streptococcal cultures into sarcoma patients and observed a cure rate of over 10%, in

what is very likely the first clinical application of cancer immunotherapy [95]. The past two decades has seen a renewed interest in the development of cancer immunotherapies invigorated by several key laboratory observations. 1) The discovery that DCs are able to present tumor associated antigens (TAAs) to the immune system [96-98]. 2) Immunodeficient mice have a higher incidence of tumors [99-101]. 3) The innate immune system is capable of immunosurveillance [102-104] all drove the pursuit of immunotherapies forward. Immunotherapies currently exist in several varieties broadly categorized into monoclonal antibody therapies, cancer vaccines and non-specific immunotherapies.

Perhaps the most success in the immunotherapy toolbox so far has come in the form of monoclonal antibody therapy. To date the US federal drug administration (FDA) has approved over a dozen monoclonal antibodies for the treatment of a variety of solid tumors and hematological malignancies [105]. Antibody therapy relies on the idea that cancer cells will have unique or over-represented antigenic epitopes allowing for their selective targeting. Antibody therapies have several routes for attacking and eliminating tumor cells. Antibodies are able to themselves directly elicit tumor cell killing by affecting intracellular signalling pathways and promoting apoptosis or through regulation of the tumor vasculature [106]. The anti CD-20 antibody rituximab is indicated for use in non-hodgkins lymphoma and promotes apoptosis in cancer cells through activation of cleaved caspases and release of mitochondrial dependant cytochrome C [107, 108]. However, most antibodies interact with the immune system and direct a cytotoxic response against the tumor through antibody dependent cellular toxicity, complement dependent-cytotoxicity and regulation of T cell function [109]. For example, ipilimumab™ functions by binding the cytotoxic T lymphocyte-associated antigen 4 (CTLA4) receptor which is a negative cell-cycle checkpoint regulator and is exploited by cancer cells to induce T cell death [110]. By effectively blocking this negative regulatory mechanism CTLA4 antibodies allow for the maintenance of proper CTL function and enhanced tumor clearance [111]. CTLA4 antibodies have shown

dramatic clinical results in a number of cancer models and represent only one example highlighting the therapeutic potential of antibody-based immunotherapies [112-114].

1.2.4.1 DC based immunotherapy

Because of their ability to elicit antigen-specific T cell responses DCs are prime candidates for anti cancer vaccine development. As previously discussed DCs are highly adept at coordinating both innate and adaptive immunity and have the capacity to capture and present TAAs to prime CTLs that specifically target tumor cells [115, 116]. Another key feature of DCs is their ability to cross-present exogenously derived antigens to $CD8^+$ T cells. The goal then for the generation of DC based vaccines is to elicit tumour-specific CTL-mediated immunity, which is sufficient and long lasting to result in tumour regression and/or eradication [6].

Despite the potential of an effective antigen-specific CTL response, DC based vaccines have met with limited clinical success. Currently Provenge™ is only FDA approved DC based immunotherapy, indicated for use in metastatic prostate cancer [47]. The effectiveness of a DC based immunotherapy is often compromised by, the limited number of available mature and functional DCs, as well as the immunosuppressive nature of the tumor microenvironment [117-119]. As discussed, DCs are only able to elicit anti-tumor immunity when they are present in a mature state. The tumor microenvironment however is highly immunosuppressive and contains high levels of Tregs, tumor associated macrophages, myeloid derived suppressor cells (MDSCs) and suppressive cytokines IL-6, IL-10 and TGF-β [120-123]. In accordance several studies revealed that there was a significant reduction in the number of mature and functional DCs that were present in tumors [124-126]. Optimal DC based vaccination strategies therefore must overcome this suppressive barrier in order to elicit sufficient immunity to induce tumor cell clearance. In order therefore for a DC based vaccine to be effective it must meet the following suggested three key criteria [4]: 1) DCs must effectively sample TAAs in the presence of sufficient cytokine signals as to induce their maturation

either encountered in vivo or delivered exogenously as part of a vaccine preparation [127, 128]. 2) DCs must migrate to the lymph nodes and prime Naïve $CD4^+$ and $CD8^+$ T cells to generate protective responses. Recruitment of innate effector cells such as NK cells would also improve anti-tumor immunity [129]. 3) Antigen-specific T cells must then overcome the immune suppressive nature of the tumor microenvironment and effectively kill the tumor. It is therefore understandable that in the face of such daunting challenges the promise of effective DC based immunotherapies have yet not garnered much clinical success.

1.2.4.2 Strategies in DC based immunotherapy

The first generation of DC based immunotherapies utilized either patient isolated or *ex vivo*-generated monocyte-derived DCs (moDCs) with little or no additional modifications. Vaccine formulations included the priming of DCs with tumor lysates, recombinant tumor antigens or synthetic peptides [130]. Despite positive outcomes with respect to patient safety these vaccines had very limited tumor regression rates. One of the first proof-of-concept clinical trials by Hsu et al. in patients with low-grade follicular B cell lymphoma highlighted the potential of tumor antigen-pulsed DCs to induce T cell specific responses [131]. A second study by Nestle et al. generated moDCs *ex vivo* in the presence of GM-CSF and IL-4 to fully differentiate DCs in contrast to the previous study, which used GM-CSF alone [132]. In this study, 5 out of 16 melanoma patients receiving treatment exhibited tumor regression further highlighting the potential for DC based vaccine formulations in eliciting anti-tumor immunity. Many of the early clinical trials involving DCs varied widely from trial to trial with respect to numbers of DCs administered, numbers of booster injections given as well as strategies relating to antigen loading. These studies representing the first generation of DC vaccines provided more of a proof of concept for the potential of DC based vaccination strategies rather than the testing of a specific therapeutic approach [133].

The development of the next generation of DC vaccines should focus on DC preparations that preferentially enhance $CD8^+$ T cell responses in combination with other therapeutic strategies that can overcome the immune suppressive nature of the tumor microenvironment. Recently the FDA has approved the monoclonal antibodies Ipilimumab and Nivolumab, which work by blocking the negative regulation of T cells through programmed death 1 (PD-1) and CTL-4 often exploited by tumors to evade the immune [134]. By combining these therapies with tumor antigen pulsed DC vaccines it may increase their anti-tumor benefits thus representing a promising new approach to enhancing T cell activation and overcoming immune suppression. Currently, there are two ongoing phase III clinical trials aimed at assessing the clinical efficacy of *ex vivo*-generated moDCs. Both of these trials are being conducted in combination with other therapies, in patients with resected tumors, and using DCs loaded with autologous tumor preparations. The first trial is being conducted in patients with newly diagnosed glioblastomas post-resection and as an add-on to standard chemotherapy and radiation regimens (NCT00045968; Northwest Therapeutics). The second study is in patients with advanced renal carcinomas using moDCs in combination with the tyrosine kinase inhibitor Sunitinib™ (NCT01582672; ADAPT trial, Argos Therapeutics). Researchers, pharmaceutical companies and patients alike are eagerly awaiting the results from these and other studies which may provide favorable outcomes where few options existed previously.

Chapter 2: PTP1B: A SIMPLE ENZYME FOR A COMPLEX WORLD

Authors: Matthew Feldhammer, Noriko Uetani, Diego Miranda-Saavedra and Michel L. Tremblay (2013). *Crit Rev Biochem Mol Biol.* **48**(5): 430-45

2.1 Abstract

Our understanding of the fundamental regulatory roles that tyrosine phosphatases play within cells has advanced significantly in the last two decades. Out-dated ideas that tyrosine phosphorylation acts solely as the “off” switch counterbalancing the action of tyrosine kinases has proved to be flawed. PTP1B is the most characterized of all the tyrosine phosphatases and it acts as a critical negative and positive regulator of numerous signalling cascades. PTP1B’s direct regulation of the insulin and the leptin receptors makes it an ideal therapeutic target for type II diabetes and obesity. Moreover, the last decade has also seen several reports establishing PTP1B as key player in cancer serving as both a tumor suppressor and a tumor promoter depending on the cellular context. Despite many key advances in these fields one largely ignored area is what role PTP1B may play in the modulation of immune signalling. The important recognition that PTP1B is a major negative regulator of Janus kinase - signal transducer and activator of transcription (JAK-STAT) signalling throughout evolution places it as a key link between metabolic diseases and inflammation, as well as unique regulator between immune response and cancer. This review will look at the emergence of PTP1B through evolution, and then explore at the cell and systemic levels how it is controlled physiologically. The second half of the review will focus on the role(s) PTP1B can play in disease and in particular its involvement in metabolic syndromes and cancer and finally we will briefly examine several novel directions in the development of PTP1B synthetic inhibitors.

2.2 Introduction

Tyrosine phosphorylation has long been established as a fundamental post-translational mechanism regulating a vast spectrum of cellular processes. This simple reaction wherein a phosphate group is either added or removed from a tyrosine residue can alter cellular signalling, proliferation, migration, and invasion. Furthermore dysregulation of this process can have broad implications for cancer, metabolism, inflammation and cardiac function. The process of tyrosine phosphorylation is orchestrated by two seemingly opposing enzymes. Initially thought of as strictly being the counterbalance to the action of tyrosine kinases, recent evidence has established the role of protein tyrosine phosphatases (PTPs) as able to both initiate and terminate cellular signalling. Despite a ten year discord between cloning of the first protein tyrosine kinase (PTK) [135] and the identification [136] and cloning [137] of the first PTP, this new field of research has advanced, uncovering 107 PTPs belonging to four distinct PTP sub-families. Interestingly, the vast majority of the members of each sub-family share a common conserved catalytic domain suggesting a single ancestral gene [138]. This review will focus on the prototypical type 1 non-receptor phosphatase (PTP1B), which has over the past decade become the subject of intense study and pharmaceutical interest initially owing to a pivotal role in leptin and insulin metabolism and more recently because it can play a dual role in human neoplastic disease [139, 140].

With over 101 cys-based PTPs, the Class 1 is by far the most studied of the four classes and can be further subdivided into “classical” and “dual specificity” phosphatases, which have the ability to dephosphorylate serine/threonine in addition to tyrosine residues. Additionally, in that subclass there is also a group of atypical PTPs that are not protein phosphatases but recognize other substrates like phosphosugars and phospholipids. The “classical” PTPs which only have affinity for phosphotyrosine residues, as dictated by the depth of their catalytic pockets [141], are further subdivided into receptor and non-receptor PTPs based on the presence of a transmembrane domain. Although several

groups were attempting to identify PTPs in the early 80's [142, 143] the first tyrosine phosphatase characterized and purified was by N. Tonks and colleagues in the late 80's [144]. This PTP was named PTP1B based on the chromatographic elution peaks identified.

Rapidly afterwards, amino acid sequence identification and cloning of PTP1B was performed by Charbonneau and Tonks [136], and the laboratory of Jack Dixon [137]. Still to this day, PTP1B remains the fundamental example of a PTP and much of the enzymology that we currently understand about PTPs comes from these earlier works. PTP1B shares many common sequence motifs with the other classical PTPs specifically with respect to their highly conserved catalytic domain. However, PTP1B as with most PTPs also contains other interaction domains that play crucial roles in defining their location, stability and function. In the case of PTP1B, besides the key catalytic domain, it also possesses two proline rich domains and an endoplasmic reticulum (ER) anchoring domain that tethers it to the cytoplasmic side of the ER allowing access to a wide range of intracellular targets [145].

PTP1B likely originated around 600 million years ago and has undergone almost perfect evolutionary conservation among all species. It has evolved a series of tightly controlled measures by which it regulates its enzymatic activity both at the transcriptional and translational level. Methods of post-translational modifications include: phosphorylation, oxidation, proteolytic cleavage and most recently sumoylation and nitrosylation. This high degree of control is essential considering PTP1B currently has over 30 known substrates and 13 interaction partners, with more suspected substrates and partners yet to be discovered [146]. Substrates include intracellular messengers involved in many cellular processes, from cell growth and proliferation to intercellular contacts and migration [147]. It is only upon closer inspection of the relatively broad range of PTP1B substrates that one can begin to appreciate how this enzyme can play

such diverse cellular regulatory activities depending on which cellular context is considered.

PTP1B was thrust into the spotlight when it was revealed that null mice were resistant to diet induced obesity and were insulin hypersensitive [148]. This seminal discovery, rapidly confirmed by the Neel's laboratory [149], led to huge interests by the pharmaceutical industry and other academic laboratories towards the screening and development of specific PTP1B inhibitors in the treatment of Type II Diabetes (T2D) and obesity. A range of compounds from natural products to competitive and noncompetitive inhibitors as well as antisense oligonucleotides have been developed, however none of which have made it past phase II clinical trials [150, 151]. A major hurdle in this regard has been high sequence homology with the closely related T cell protein tyrosine phosphatase (TC-PTP) and other PTPs. Despite this, a wealth of publications describing the multiple functions of PTP1B has appeared in the literature in the last 20 years.

This review will focus on the evolution, regulation and role(s) of PTP1B in diseases. In particular we will examine where PTP1B appears along the evolutionary timeline, and examine its structure, catalytic mechanism and common means of regulation. We will then draw attention to some of the most recent and significant works looking at how PTP1B can regulate metabolism. In particular, insulin and leptin signalling will be discussed. PTP1B's involvement in controlling the ER stress response and how this relates to obesity and inflammation will also be considered. We will also briefly emphasize the novel findings regarding PTP1B in breast and prostate cancer. Finally, how PTP1B can control signalling pathways in the field of immunology will be examined. Notably, we included some thoughts on PTP1B's influence on B cell development, macrophage regulation and highlight several commonalities in PTP1B activity that links these together.

2.3 Origin and evolution of the *PTPN1* (PTP1B) gene.

Bioinformatic analyses using the Ensembl Compara Gene Tree visualization tool [152] and the MetaPhOrs database [153] suggest that the *PTPN1* gene, which encodes the PTP1B enzyme, is conserved from Placozoans to human in 1:1 orthology relationships. Whereas EnsemblCompara GeneTrees only considers vertebrate species plus a few key non-vertebrate model organisms (such as the fly, the worm and baker's yeast) [154], the MetaPhOrs database is a comprehensive resource that combines evidence from multiple and independent sources (PhylomeDB, Ensembl, TreeFam and Fungal Orthogroups databases, plus those reconstructed for EggNOG, OrthoMCL, and COG). An important advantage of MetaPhOrs is that it provides consistency scores for the orthology and paralogy predictions for 829 sequenced genomes by using information from the multiple methods, which can be used as more reliable confidence scores. Examination of *PTPN1* homologous genes allows us to draw the following conclusions: (i) a single, ancestral, *Ptpn1*-like gene orthologous to present-day PTPN1 first appeared prior to the emergence of the lineage leading to Placozoans, such as *Trichoplax adhaerens*. Placozoans are a basal group of multicellular animals lacking true tissues, organs, body symmetry, or identifiable internal structures. Although *T. adhaerens* moves by means of cilia and ingests microbes by absorption, its ~100 Mb genome is surprisingly complex: 90% of the ~11,500 genes are found in other animals (with the positions of introns largely conserved); and the genome harbors a surprisingly complex array of transcription factors and signalling pathways that we normally associate with complex developmental patterning and cell type-specification programs in higher metazoans. Placozoa likely diverged about 600 million years ago, and we may place the emergence of the *PTPN1* gene at this point in the evolutionary timescale (**Figure 2.1**) [155]. (ii) The emergence of the *PTPN2* gene likely happened much later, following a gene duplication event of the ancestral (*PTPN1*-like) gene before the fish evolutionary split. All genomes analyzed harbor one copy of the *PTPN1* gene, even in fishes which are known to have undergone a fish-specific genome duplication event ~350 million years

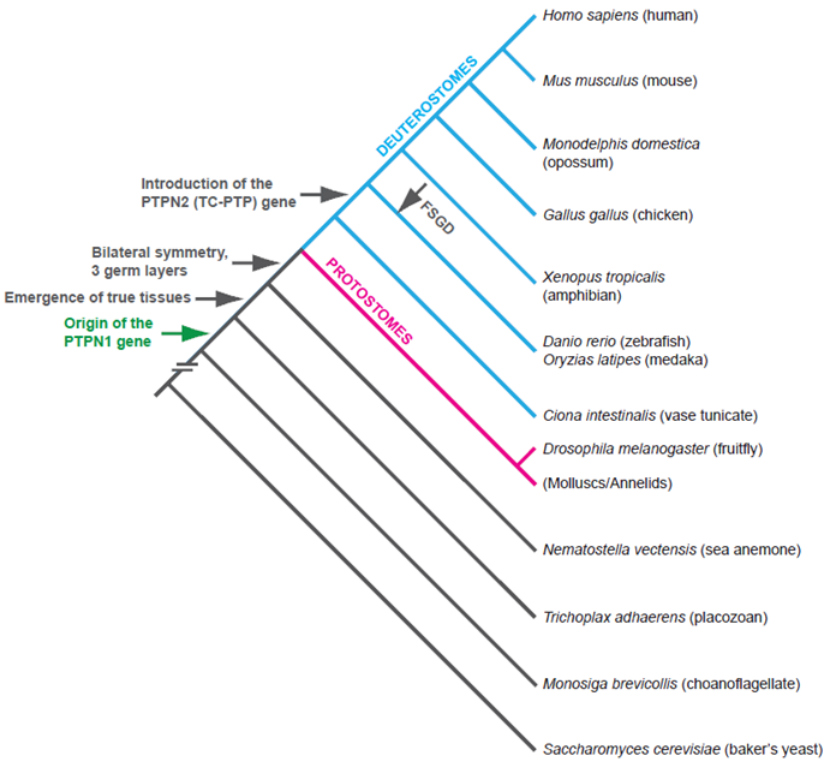

Figure 2.1 Evolutionary history of the PTPN1 gene. The PTPN1 gene first arose ~600 million years ago before the split of the lineage leading to the present-day placozoan *Trichoplax adhaerens*, a multicellular organism with a complex genome but without true tissues, organs or body symmetry and believed to be basal to the rest of extant animals.

ago [156]. Although the duplication of genes and entire genomes is an important mechanism for the evolution of phenotypic complexity, most duplicated genes are usually lost secondarily, which was probably the fate of the duplicated *Ptpn1* gene in the fish lineage.

2.4 Genomic organization of the *PTPN1* gene.

PTP1B was originally purified as a truncated catalytic domain from human placenta almost 25 years ago [136]. The 50 kDa protein was subsequently found to be encoded by the *PTPN1* gene [157], which upon translation produces a 435 amino acid protein with a 35 amino acid C-terminal tail that targets PTP1B to the cytoplasmic face of the endoplasmic reticulum (ER). The human gene (located to band 20q13) spans over 74 kb with an unusually large first exon. The organization of human and mouse genes is identical except for an additional exon at the 3' end of the human ortholog which is absent from the mouse gene (ten exons in human for nine in the mouse) [158]. Interestingly, this locus is frequently amplified in breast cancer and associated with poor prognosis [159]. Furthermore, the *PTPN1* locus is linked to T2D [160], fat mass [161] and energy intake [162], thus supporting a role for *PTPN1* in cancer and metabolism.

2.4.1 The ten motifs of the PTP catalytic domain and their involvement in catalysis

Like all members of the PTP family, the catalytic domain of PTP1B harbors ten conserved motifs. Motif no. 9 represents the catalytic motif [I/V]HCXXGXXR[S/T] [141], which has been perfectly conserved from the very primitive *T. adhaerens* to human (**Figure 2.2**). The ten motifs of the PTP catalytic domain act in coordination during the two-step catalytic process [163, 164]. Briefly, the PTP-loop (motif no. 9) contains the catalytic cysteine (residue 215 in human PTP1B) that is responsible for executing the nucleophilic attack on the substrate phosphate moiety. The catalytic pocket is highly acidic (pKa approx 5.4) due to the surrounding chemical environment, thus enhancing the nucleophilic properties of the active site cysteine while making it more prone to oxidation, an important modification which will be discussed below [165]. The second

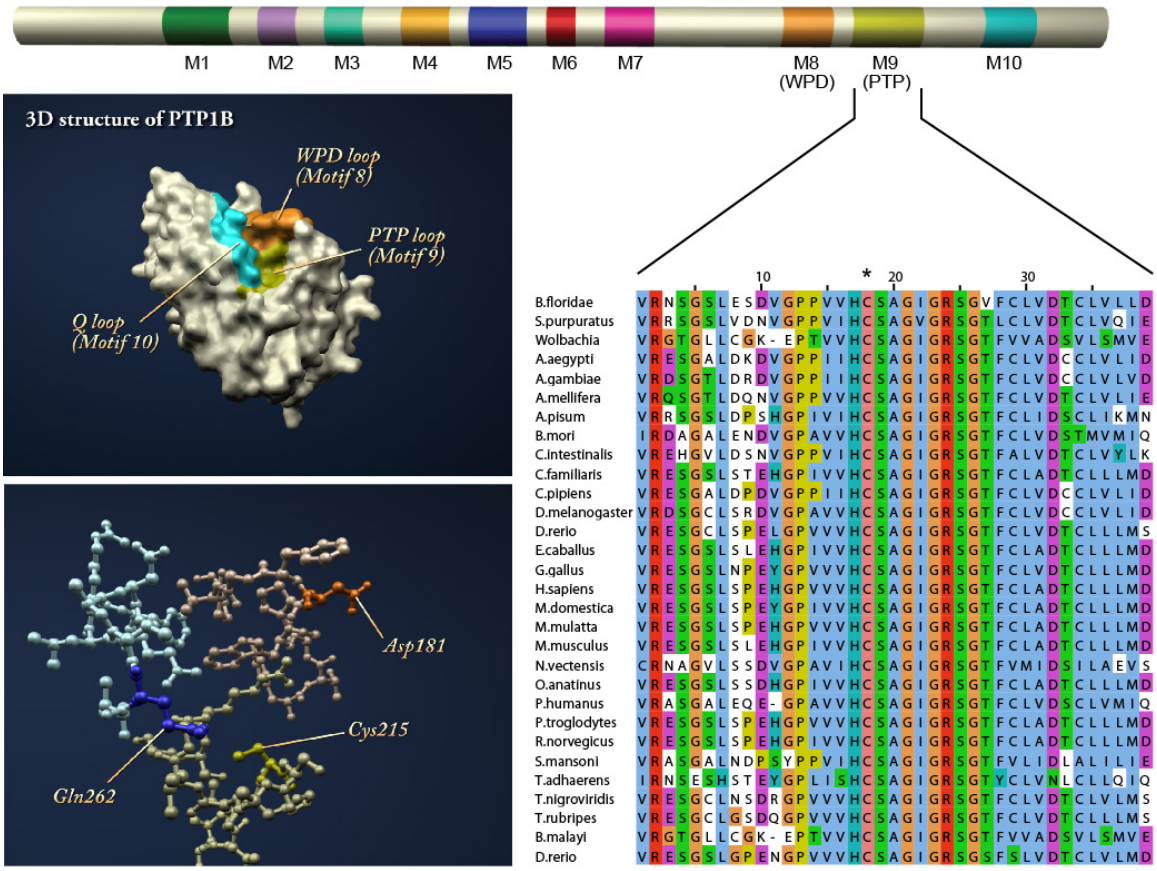

Figure 2.2 Schematic illustration of the PTPN1 gene and the 3D structure of PTP1B. Like all tyrosine-specific phosphatases, the PTP1B enzyme harbors ten clearly identifiable motifs. Motif no. 9 contains the highly conserved catalytic cysteine residue, which is remarkably well conserved across all species investigated. The 3D structure of PTP1B is shown on the left, with the critical motifs for the enzymatic reaction indicated in color to highlight the catalytic pocket where substrate phosphate moieties bind. A ball-and-stick model illustrates the critical catalytic residues in the secondary structure: Cys 215 (yellow) Asp 181 (orange) and Gln 262 (blue). Note the position of the Asp residue, which upon substrate binding swings over the pocket, thereby locking the enzyme in the closed conformation during catalysis.

step of the reaction is carried out by the WPD loop (motif no. 8). The WPD loop is a highly flexible motif, which permits the active site to function in either an “open” and “closed” conformation. In its “open” conformation the WPD loop moves out of the way, thus rendering the catalytic pocket accessible to substrates. Upon substrate binding, the loop swings back over the catalytic pocket bringing the aspartate residue in proximity for catalysis. The last step of the reaction involves the Q loop (motif no. 10) where glutamic acid residue 262 of the Q loop and the aspartic acid residue 181 of the WPD loop act in concert to hydrolyze the cystinyl-phosphate intermediate through the coordination of a water molecule in a general base reaction freeing the phosphate from the active site (this enzymatic process is depicted in Movie 1) [166, 167].

2.4.2 Importance of the non-catalytic motifs of PTP1B.

PTP1B harbors two proline-rich motifs and an ER-targeting motif that play important roles in its regulation. The proline-rich motifs (amino acids 300-308 and 309-314 in human PTP1B) has been reported to mediate the binding of key SH3 domain-containing proteins such as $p130^{Cas}$, Grb2, Crk and Src [168]. A more recent report established that the proline-rich motif PPRPPK (residues 309-314) is essential for the establishment of a PTP1B-Src-$p130^{Cas}$ complex that links PTP1B to integrin-mediated signalling and cell migration [169]. This finding is important as it establishes a clear link between PTP1B and integrin signalling that controls cell-cell contacts, cell migration and invasion, all of which are key metastatic properties of tumors

PTP1B localizes to the cytoplasmic face of the ER membrane by virtue of a C-terminal 35 amino acid hydrophobic tail [170], which in principle also serves as a regulatory mechanism by limiting the cellular space where PTP1B can exert its enzymatic activity [171]. Recent evidence, however, argues that despite its subcellular localization, PTP1B is unconstrained with regard to its ability to dephosphorylate intracellular substrates [172]: although the predominant form of PTP1B is localized to the cytoplasmic face of the ER, calpain-mediated cleavage has been shown to free PTP1B from this cellular

niche into the wider cytoplasmic space with enhanced catalytic activity [173]. PTP1B may thus exert its enzymatic function despite its ER localization in four distinct ways [174]: (i) calpain-mediated cleavage frees PTP1B from the ER [173]; (ii) activated receptors are endocytosed at the plasma membrane upon ligand stimulation and come in close proximity to the ER [145, 175]; (iii) the ER network itself can stretch out and come into direct contact with the plasma membrane [174], and (iv) PTP1B can act locally on RTKs during their biosynthesis within the ER [176]. Additionally, a recent report shows that PTP1B can localize to the inner nuclear membrane where it interacts with emerin in a cell cycle-dependent fashion, a novel finding which further expands the pool of substrates available to PTP1B [177].

2.4.3 Transcriptional and post-translational regulation of PTP1B.

Since PTP1B regulates a vast array of signalling pathways, its function must be under tight regulation to avoid aberrant cellular signalling. The regulation of PTP1B, whether by PTMs or at the level of transcription, has been extensively studied since the discovery of the enzyme (reviewed in [140, 141]). Below we will briefly discuss some of the key findings in this domain as well as highlight some of the more recent work in the field.

The expression of PTP1B is regulated by early growth response-1 and the Sp family of proteins [178], Y box binding protein 1 [179], NF-κB [180]. More recently, we showed that when prostate cancer cells are stimulated with androgen, the androgen receptor is recruited to the promoter of *PTPN1*, leading to an increased recruitment of RNApol II to the transcription start site [181]. Taken together, these findings unveil a complex interplay of transcriptional regulatory mechanisms that exerts a tight control over PTP1B in a context-specific manner.

PTP1B is the subject of numerous PTMs, including phosphorylation, oxidation, nitrosylation, sumoylation and proteolytic cleavage. PTP1B is phosphorylated on serine and tyrosine residues in response to insulin, EGF, and PDGF stimulation, which can

either enhance or abrogate its enzymatic activity [182-184]. Several phosphorylation sites have been identified in large-scale mass spectroscopy studies [185-187] but a detailed understanding of how each phospho-residue contributes to the regulation of PTP1B is still lacking. Dadke and co-workers showed that PTP1B can also be sumoylated on Lys335 and Lys347 [188]. This modification results in decreased activity towards IR signalling and also affects the PTP1B-mediated dephosphorylation of the key nuclear architectural protein emerin [177].

Calpains are ubiquitously expressed cysteine proteases that can mediate the inactivation and degradation of cellular PTPs [189]. In the case of PTP1B, calpains are also capable of cleaving its ER-targeting domain. In activated platelets, this results in a 42kDa soluble form of the PTP1B enzyme with its catalytic activity increased two-fold, suggesting a role for the ER targeting domain in negatively regulating this enzyme [173]. Knockout (KO) mouse studies demonstrated that Calpain-1 was shown to specifically target PTP1B, which is required for proper activation of the platelet aggregation and clot retraction pathways [190, 191]. Additionally, Calpain-2 and PTP1B synergistically function in a novel pathway with Src to regulate invadopodia dynamics and breast cancer cell invasion [192]. Finally, upon UV-induced oxidation of the catalytic residue Cys215, PTP1B exhibits an increased association with calpains, which inactivate PTP1B by cleavage occurring predominantly at Ala76 [193].

PTPs are highly sensitive to reactive oxygen species (ROS) because of the acidic environment within their catalytic clefts [165, 193]. One of the main cellular consequences of PTP1B inactivation by ROS is altered immune signalling and cytokine crosstalk [194]. Oxidative modifications of the active site cysteine generally result in the abrogation of catalytic activity since PTP1B can no longer execute a nucleophilic attack. This modification can either be permanent or transient, depending on whether the enzyme is converted to the reversible sulphenamide (S-OH) state, or the irreversible sulphinic ($S\text{-}O_2H$) and sulphonic ($S\text{-}O_3H$) forms [195, 196] as is the case when using the

pan-tyrosine phosphatase inhibitor vanadate [197]. Reactive nitrogen species much like ROS have been shown to inactivate PTPs [198, 199]. Specifically, the S-nitrosylation modification of the active site cysteine of PTP1B as mediated by NO was demonstrated to play an important protective role against irreversible H_20_2-induced oxidation [200]. Lastly, a recent study exploited the conformational changes that the catalytic cysteine Cys215 typically undergoes upon reversible oxidation by generating antibodies that maintain PTP1B in an inactive state. These conformation-sensing intracellular antibodies "intrabodies" stabilize the oxidative form of PTP1B and effectively inhibit its enzymatic activity. This exciting new tool has potentially very important therapeutic applications [201].

The examples presented in this section illustrate some of the myriad ways in which the activity of PTP1B can be regulated, which can have vastly divergent consequences depending on the stimulus and cellular context. Therefore, PTP1B is a highly dynamic enzyme capable of regulating cellular signalling in many contexts with exquisite precision.

2.5 Experimental identification of PTP1B substrates.

Much of what we know about PTP1B comes from the identification of its diverse set of substrates, including over 30 target phosphoproteins and 13 interacting partners [146]. PTP1B interacts with a wide array of proteins responsible for numerous cellular processes, including the insulin receptor (IR) [182, 202], the epidermal growth factor receptor (EGFR) [203] and the JAK-STAT transducers [204, 205]. More recently STAM2 [206], cortactin [207] and PKR-like eukaryotic initiation factor 2α kinase (PERK) [208] have also been identified as direct substrates of PTP1B. By exploiting and understanding the critical residues responsible for the catalytic reaction mechanism, researchers were able to develop trapping mutants for the identification of these target substrates. Initially the active site cysteine or the invariant aspartic acid residues were mutated to serine or alanine, respectively [209]. These modifications allowed the

normally rapid and transient association of the substrate with the enzyme to become stabilized to the point where substrate identification became possible as the enzyme was no longer catalytically active, or was severely impaired with respect to hydrolyzing the bound phosphate. Subsequently researchers improved upon these initial trapping mutants by either combining them to make double traps (C215S-D181A or D181A-Q262A) or identifying novel sites that would enhance substrate trapping ability [210, 211]. Recently Boubekeur *et al.* further improved the established D/A trap by introducing the Y46F mutation, a residue of the pTyr recognition loop (motif no. 1) [212]. This crucial tyrosine residue is responsible for defining the depth of the catalytic pocket, thus conferring increased substrate specificity to PTP1B by not allowing the shorter phosphoserine or phosphothreonine to reach down deep enough to the active site [166, 213]. This novel trapping vector effectively increased the total amount of phosphotyrosine proteins that were trapped by the PTP1B mutant, thus becoming a more powerful tool for the identification PTP1B substrates.

2.6 PTP1B in metabolism and insulin signalling.

Initial *in vitro* evidence suggested a function for PTP1B in activating the IR and its downstream signalling pathways [144]. Vanadate (which has been used for over a century in diabetes treatment) was later identified as a pan-PTP inhibitor [214], adding further support for the role of PTP1B in regulating the IR [215]. Confirmation of the importance of PTP1B in metabolism came from PTP1B null mice, which appear healthy although they present slightly lower blood glucose levels as well as significantly lower insulin levels when compared with the wild type (WT) littermates. On a high-fat diet the PTP1B KO and heterozygous mice were resistant to weight gain and remained insulin-sensitive unlike the WT mice, which rapidly gained weight and acquired insulin resistance [148]. At the molecular level, PTP1B KO mice present increased phosphorylation of the IR in liver and muscle tissue upon insulin injection, thereby firmly establishing PTP1B as a key regulator of insulin signalling. This study sparked a rapid drive to develop PTP1B inhibitors by the pharmaceutical industry for the treatment of

obesity and T2D, and the initial observations were quickly reproduced [149]. Proof of concept for pharmaceutical intervention was provided by scientists at Abbot when an antisense PTP1B oligonucleotide normalized glucose levels and improved insulin sensitivity in diabetic mice [216]. Further studies using tissue-specific models of PTP1B ablation revealed that despite improved glucose homeostasis and insulin signalling in muscle [217] and liver [218] lacking PTP1B, it was only through the deletion of PTP1B in neurons that mice were protected from diet induced obesity and glucose intolerance [219].

Recently, Yang *et al* showed that the dysregulation of liver microRNA miR122 contributes to hepatic insulin resistance through the induction of PTP1B via a c-Jun N-terminal kinase 1 (JNK1)- hepatocyte nuclear factor 4α axis [220]. This finding represents a fresh insight into the *in vivo* regulation of PTP1B, and establishes PTP1B as a direct target of miR122. Studies in flies unveiled a novel mechanism for the down-regulation of the IR by PTP1B and necessarily mediated by Dock (Nck1 in humans). The requirement of the adaptor protein Dock for the efficient dephosphorylation of the IR both *in vitro* an *in vivo* underlines the role of adaptor proteins in conferring PTP substrate specificity [221]. Of note, obese mice with livers deficient in Nck1 display attenuated ER stress signalling and improved glucose tolerance and insulin signalling through attenuated inositol-requiring enzyme 1 α (IRE1α)-JNK activation [222]. It has also been shown that IRE1 signalling is specifically inhibited by PTP1B [223], suggesting a mechanism whereby Nck1 modulates IRE1α signalling through PTP1B by linking insulin receptor signalling with the ER stress response [222].

2.7 Role of PTP1B in leptin regulation.

Leptin is a key hormone that regulates energy intake and expenditure, including appetite/hunger, and a wide array of metabolic and energy regulatory processes. Leptin is directly associated with obesity in mammals [224]. Knockout mice provided the first evidence that PTP1B attenuates leptin signalling by dephosphorylating the downstream

effector Janus kinase JAK2. Mice deficient in leptin and PTP1B display reduced weight gain, lower amounts of adipose tissue and increased resting metabolic rates [225, 226]. These dramatic results underlie a PTP1B-mediated mechanism for the control of diet-induced obesity specifically mediated by the deletion of PTP1B in neurons [219]. In the brain, two distinct populations of first-order neurons synthesize either agouti-related protein (AgRP) or proopiomelanocortin (POMC) which have opposite effects on energy balance [227, 228]. Mice with a POMC neuron-specific deletion of PTP1B exhibited reduced adiposity, improved leptin sensitivity and increased energy expenditure compared with WT controls [229] However, despite a central role for POMC neurons in controlling food intake, there were no differences in this regard between WT and PTP1B knockout animals, suggestive of a role in other neuronal populations for PTP1B's ability to regulate food intake [229, 230]. Recently, it was shown that the deletion of PTP1B in neurons expressing the leptin receptor resulted in leptin hypersensitivity and reduced body weight on regular as well as high-fat diets, thus highlighting the role of leptin receptor-specific neurons as the key to understanding the phenotypes seen in whole body PTP1B knockout mice. Still, subtle differences between leptin receptor-specific versus global PTP1B knockouts, (such as the difference in adiposity on normal chow diet) suggests that there remains a leptin-independent metabolic role for PTP1B to be discovered [231].

Leptin is also involved in the control of cardiovascular function via regulation of the sympathetic tone, which is responsible for vasoconstriction and dilation [232-234]. Since leptin signalling is regulated by PTP1B, and obesity is a risk factor for cardiovascular disease, it was hypothesized that PTP1B could play a role in regulating cardiovascular function. Indeed, PTP1B deficient mice have higher blood pressure (BP), and leptin infusion further increases BP [235]. Further support for a role of PTP1B in regulating cardiac function comes from the observation that the deletion of PTP1B improved vascular dysfunction in obese mice [236]. Another study assessed the role of PTP1B in chronic heart failure and concluded that inhibition was beneficial through increased left

ventricle function and decreased adverse remodeling possibly leading to PTP1B acting as a new therapeutic target for chronic heart failure [237]. Additional important findings include a role for the microRNA miR210 in regulating PTP1B, specifically with respect to delaying apoptosis and improving heart function following myocardial infarction [238]. Finally, PTP1B also acts in angiogenesis via the regulation of VEGFR2, where it stabilizes cell-cell adhesions by reducing the cellular tyrosine phosphorylation levels of VE-cadherin [239], a remarkable observation with important consequences for cardiac function and cancer metastasis.

2.8 PTP1B in endoplasmic reticulum (ER) stress.

Over the past ten years a critical link has been established between ER stress and metabolic diseases, particularly obesity and T2D. This relationship was first proposed by the pioneering work of Ozcan *et al* who showed that ER stress is a central factor underlying how obesity leads to insulin resistance and T2D, both in cellular and mouse models [240]. ER stress is caused by the accumulation of improperly folded proteins within the ER lumen. The ER chaperone binding immunoglobulin protein (BiP) binds to and becomes sequestered by such proteins, resulting in the activation of three sensors and their downstream pathways, which are cumulatively referred to as the unfolded protein response (UPR). These sensors (IRE1, activating transcription factor-6 (ATF6) and PERK) fine-tune the rates of protein synthesis, apoptosis and folding capacity in order to maintain cellular homeostasis through the induction of translational and transcriptional changes. For instance PERK phosphorylates eukaryotic initiation factor 2-alpha (eIF2α) to attenuate global protein synthesis [241, 242]. Gu *et al* established a direct link between PTP1B and the ER stress response by showing that PTP1B-null cells are resistant to IRE1-mediated ER stress signalling pathways, therefore establishing a direct link between PTP1B and the ER stress response [223]. To further explore this novel role for PTP1B in regulating the ER stress response, tissue-specific null mouse models have been used. Two studies utilizing a liver-specific deletion of PTP1B showed decreased ER stress response in mice on a high fat diet. These mice presented decreased signalling in

UPR pathways, specifically the liver p38 MAPK, as well as attenuated phosphorylation of JNK, pPERK, and eIF2α [218, 243] These results highlight how PTP1B can regulate the ER stress response through a variety of mechanisms and provide evidence that disrupting PTP1B in the liver can alleviate ER stress induced by high fat diet and obesity.

Recent work by Tonks and colleagues revealed yet another role for PTP1B in regulating the UPR, whereby ER stress induced production of H_2S, thus inactivating PTP1B via sulfhydration of the active site cysteine residue. This transient inhibition of PTP1B protects the activation domain of PERK, allowing it to maintain eIF2α in an inactive state and thus suppressing global protein translation [208]. This study also established PERK as a substrate of PTP1B, which acts on $pTyr^{619}$. Another group explored the interplay between PTP1B and PERK in brown adipocytes (a tissue with anti-obesity properties responsible for generating heat via mitochondrial uncoupling of lipid oxidation) [244, 245]. The primary finding was that brown adipose tissue and adipocytes lacking PTP1B presented an enhanced PERK/eIF2α activation of the UPR. This is in line with their previous findings whereby PTP1B deficiency leads to increased PERK/eIF2α signalling in pancreatic beta cells that have been exposed to chemical stress [246]. Additionally, the authors confirmed previous findings by Krishnan *et al* [208] that PERK is regulated by PTP1B at $pTyr^{615}$ (the human residue orthologous to Y619 in murine PTP1B). It is important to note that these reports are in contrast to the previously mentioned results (Gu et al., Delibegovic et al. and Agouni et al.) which suggest that, like the differential regulation of lipogenic genes in hepatocytes and adipocytes in response to protease inhibitors, PTP1B may regulate the same pathway in a tissue-specific manner, resulting in different signalling and biochemical outcomes [244]. More recently, examination of the interplay between PTP1B and ER stress in cultured myotubes showed that PTP1B expression levels are increased in these cells in response to tunicamycin, and that similar treatment of the PTP1B KO mice also resulted in attenuated eIF2α and JNK2 phosphorylation [247]. These findings that point to a requirement for PTP1B for activating PERK are consistent with those of Bettaieb *et al* [244], and in opposing

contrast to those of Gu *et al.* (Gu et al, Delibegovic et al and Agouni et al). However, the authors stipulate that their *in vivo* results were obtained in muscle homogenates and not with isolated myotubes. Therefore the contributions of other tissues towards the effects observed cannot be ruled out [247]. Another potential explanation might be there are different kinases (for example Src family kinases) upstream of PERK that may be activated by PTP1B. These seemingly contradictory conclusions on the role of PTP1B in regulating ER stress still highlight an important theme that underlies much of the research on PTP1B, which is the highly context specific nature with which PTP1B can modulate cellular signalling. Additional studies will be required to fully comprehend how PTP1B can regulate ER stress and explain its influence on obesity and T2D (**Figure 2.3**).

2.9 Link between metabolism and immunity.

A growing amount of evidence suggests that obesity should be regarded as an inflammatory state to the extent that it is now considered a field of research in its own right [248]. Immune cytokines are part of the body's response to obesity-induced metabolic changes. These include pro-inflammatory cytokines TNFα, IL-1, IL-6 as well as cellular changes like leukocyte activation and local recruitment to inflamed tissue [248-250]. Clear links between PTP1B and several of these immune pathways have been established, including the regulation of the JAK-STAT pathway [204], as well as TNFα [180, 251] and IL-6 signalling [251, 252]. Therefore it is reasonable to assume a dual role for PTP1B in regulating obesity directly through its action on both IR and leptin signalling, and indirectly through of the modulation of the inflammatory state and recruitment of immune cells.

2.10 PTP1B genetic variants.

A number of genome-wide association studies (GWAS) have suggested an association between the *PTPN1* locus with T2D, although numerous other reports have challenged this association. For instance, three distinct studies examining either Caucasian [253] Hispanic Americans [254] or Canadian Oji-Cree [255] populations reported a positive

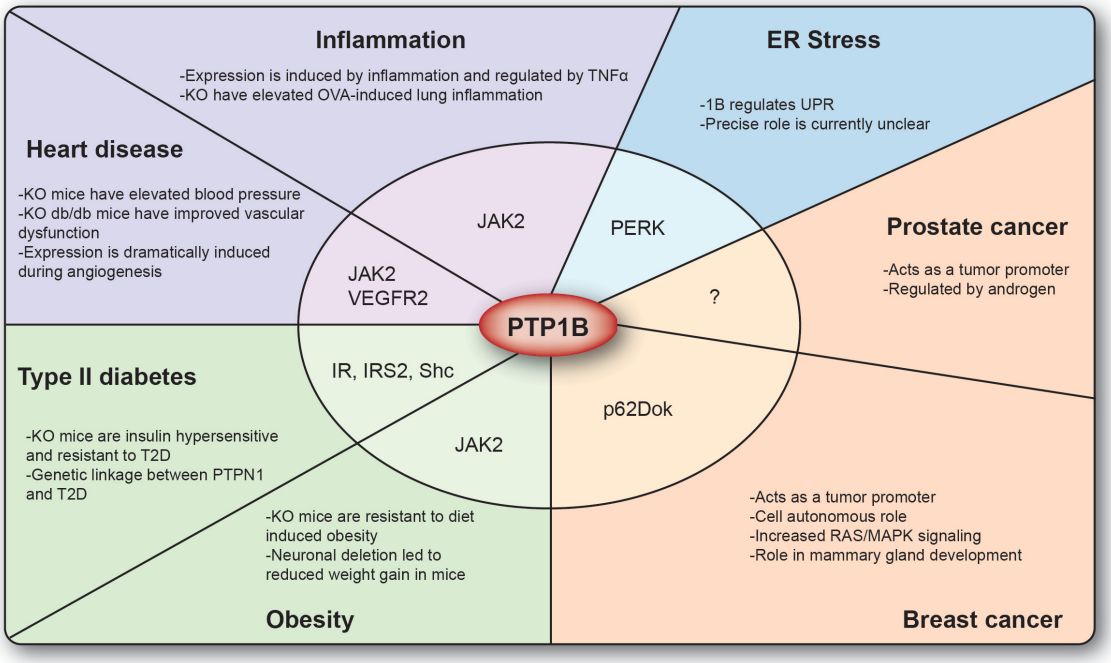

Figure 2.3 Overview of PTP1B in disease. Illustration outlining several key findings relating PTP1B to disease states, together with the known substrates wherever known. PTP1B has been shown to play a prominent role in metabolic signalling (especially obesity and T2D) through interaction with the IR and JAK2 downstream of the leptin receptor. Additionally, PTP1B through p62Dok and a still to be discovered substrate has pro-tumorigenic properties both in breast and prostate cancer. A significant amount of evidence has accumulated linking PTP1B to the ER stress response through interaction with PERK, although the effect of this interaction is still debated. PTP1B can also influence inflammation through regulation of the various JAKs and STATs, suggesting an interesting and still largely undiscovered role in immune signalling.

association between specific single nucleotide poylmorphisms (SNPs) in the *PTPN1* locus and either susceptibility to [253, 254], or protection from T2D [255]. On the other hand, two additional studies conducted in a European sample group reported no association between *PTPN1* polymorphisms and T2D [256], although several SNPs of *PTPN1* were subsequently (but weakly) associated with obesity [257]. Among the polymorphisms reported in the *PTPN1* locus, the P387L mutation was described in a Danish sample of 527 cases as being associated with T2D [258]. Interestingly the authors demonstrated that this mutation impairs the ability of a PTP1B peptide to undergo *in vitro* phosphorylation on the adjacent serine, which is targeted by $p34^{cdc2}$ during mitosis [259]. This mutation, however, was not found to be associated with T2D in an analysis of Chinese or Finnish patients [260, 261]. Overall, the contradictions found in these reports are likely due to the high heterogeneity of the underlying causes among diabetic patients, or the inherent diversity of the cohorts themselves, therefore making it impossible to draw any definitive conclusions at this stage.

2.11 PTP1B in cancer.

The role of PTP1B in cancer has been extensively reviewed elsewhere [140, 262, 263], and here we will briefly discuss the most recent findings. Since many PTKs have oncogenic properties, it would be reasonable to assume that many PTPs function as tumour suppressors. The tumor-suppressing properties of PTP1B were initially ascribed to observations from cell culture experiments where it was shown that overexpression of PTP1B protects cells from transformation and decreases tumorigenicity in *v-src* (reversion of phenotype) [264] and *neu* [265] transformed cells. Additionally, PTP1B can suppress p210 bcr-abl transformation in Rat-1 fibroblasts in contrast to TC-PTP, which lacks this property. Despite these initial findings, we know that PTP1B null mice do not develop spontaneous tumors nor have increased tumor incidence, and that in fact PTP1B can be tumor-promoting depending on the cellular context [266, 267].

2.11.1 PTP1B in breast cancer and breast development.

Much of the recent work in the field has focused on the role of PTP1B in breast cancer by studying whole body and tissue-specific PTP1B knockouts. Early studies reported the overexpression of PTP1B in 72.4% of human tumor sections (n=29) at various developmental stages, thus lending support to an important role for PTP1B in the development of breast cancer [268]. Further understanding of the importance of PTP1B in breast cancer came from work in our laboratory that reported using a transgenic expression model that PTP1B alone, when expressed via the MMTV promoter, was able to initiate transformation [267]. When the PTP1B KO mutation was introduced into a MMTV-EBRB2 mouse model, the result was a significant delay in transformation. In part the delay in cancer growth could be linked to the p62dok and RAS interaction complex, which decreased MAPK signalling and proliferation [267]. Remarkably, this delay in tumorigenesis was also reproduced in the transgenic MMTV-ERBB2 mice using a small molecule inhibitor of PTP1B, and a two-week treatment with such inhibitor led to over a month of protection. These findings alluded to the possibility that PTP1B was not just synergizing with the ErbB2 oncogene, but that its inhibition promoted a systemic and long-lasting anticancer effect even when using its inhibitor molecule. Similar findings on the delayed tumorigenicity in MMTV-ErbB2 PTP1B-deficient mice were independently reported by Ben Neel's group [269]. Finally, another signalling cascade linking ErbB2-PTP1B-Src kinase was later found to contribute to the oncogenic properties of PTP1B in human breast epithelial cells [270]. A follow-up study by the Bentires group using tissue-specific and inducible KO PTP1B models showed that PTP1B acts in a cell-autonomous manner in the mammary epithelium to delay Her2 tumors, and that PTP1B is not involved in tumor maintenance or growth once it is established [271]. The same group recently identified PTP1B as a fundamental player in mammary gland development and remodeling (alveologenesis and lactogenesis), where the deletion of PTP1B enhanced progesterone receptor expression and phosphorylation of STAT5 (a key transcription factor in mammary development). Additionally, the mammary glands of knockout mice produced more milk during pregnancy due to enhanced STAT5 activation. PTP1B

deletion also increases the number of mammary progenitor cells and induces early formation of alveoli. This occurs in part through the reduced expression of the estrogen-responsive genes progesterone and Rankl, which are critical in the formation of mammary alveoli and expansion of mammary stem cells [272, 273]. This study offers an explanation for the protection offered by PTP1B in breast cancer in KO mice by showing that PTP1B deletion induces the early differentiation of the mammary gland, which may lead to decreased numbers of Her2 transformed cells [274]. Of note, a recent publication analyzed a significant number of tissue samples (n=1402) and showed that no correlation exists between PTP1B and HER2 expression, or PTP1B and age, tumor size, tumor stage, histologic grade, lymph node status, or histological subtype. However, PTP1B expression was associated with estrogen receptor expression and intrinsic molecular subtype, and it was proposed as an independent predictor of improved survival in breast cancer [275]. These findings contradict all previous murine and histological studies which portray PTP1B as a tumor promoter in the breast, and therefore specific additional investigations that will clear this ambiguity are needed before considering PTP1B in a therapeutic context in breast cancer.

2.11.2 PTP1B in prostate cancer.

Signalling pathways of specific importance in prostate cancer are known to be regulated by PTP1B, including EGFR, PDGFR, c-Met, IGF-R1, Src and various JAKs, and the PI3K-Akt and MAPK signalling cascades [276, 277]. Furthermore, there is strong evidence linking diet, obesity and prostate cancer and the role of PTP1B in these diseases is already well established, thus providing another potential association. Initial *in vitro* studies showed that overexpression of PTP1B can lead to neuroendocrine differentiation, and that androgen withdrawal in prostate cancer cells (LNCaP) leads to increased expression of PTP1B, providing the first evidence for a role of the phosphatase in this disease [278]. Building from this early evidence and the strong correlations mentioned above, our group showed that PTP1B expression is increased in human prostate cancer tissues and is directly regulated by the androgen receptor. Using a combination of methods we

demonstrated that PTP1B promotes prostate cancer cell migration and invasion *in vitro*, and is associated with tumor growth and metastasis *in vivo* due in part to an unidentified substrate of PTP1B [181].

2.11.3 The role of PTP1B in cancer and immunity.

Initial investigations into the roles of PTP1B in cancer were naturally followed by the crossing of PTP1B knockout mice with the well-established P53 knockout tumor model. Unlike PTP1B knockout mice, P53 null mice develop spontaneous tumors [279], especially T cell lymphomas [279, 280], but upon crossing the two models, double knockout mice ($p53^{-/-}$ $PTP1B^{-/-}$) exhibited decreased survival and earlier tumor development compared with PTP1B WT and heterozygous mice [266]. The double knockout mice also developed lymphomas with a particular bias towards B cell as opposed to T cell lymphomas, suggesting a role for PTP1B in regulating hematopoietic function. Additionally $p53^{-/-}$ $PTP1B^{-/-}$ mice have increased numbers of B cells in the bone marrow and lymph nodes, and larger numbers of B cell progenitors also in the bone marrow too. These findings highlighted an important role for PTP1B in regulating latency and tumor type in P53 null mice as well as a role in B cell development [266]. Additionally, another report demonstrated elevated PTP1B expression in activated B cell–like diffuse large B-cell lymphomas (DLBCL), as well as several other non-hodgkins lymphomas: PTP1B expression was observed in 58% of DLBCL, 62% of peripheral T-cell lymphomas, and 33% of marginal zone lymphomas. PTP1B expression was also correlated with the expression patterns of lymphoma-specific markers BCL2 and MUM1. Interestingly, this same report proposed that PTP1B regulates phospho-STAT6, and demonstrated that IL-4 induced PTP1B expression in a PI3K dependent fashion, IL-4 plays an important role in neoplastic, and hematological malignancies [281]. Taken together, these findings suggest a fundamental role for PTP1B in the regulation of immune cell signalling and hematopoietic cancers.

2.12 Emerging roles of PTP1B in regulating immune signalling.

As discussed above, PTP1B plays critical roles in the regulation of metabolic signalling through the IR and the leptin receptor-associated JAK2. Recent evidence has established that obesity can be considered an inflammatory condition, thus bridging the fields of immunology and metabolism [248]. In fact, some of the most extensively studied substrates of PTP1B are the critical immunomodulators within the JAK-STAT pathway, where PTP1B specifically targets JAK2 and TYK2. This supports a role for PTP1B as a genuine modulator of immune signalling [204, 281, 282].

2.12.1 Myeloid cells: monocytes, macrophages and granulocytes.

Myeloid precursors give rise to important mediators of the innate immune system, including macrophages, granulocytes and dendritic cells. Myeloid development is in part dependent on the JAK2-mediated signalling of cytokines IL-3, granulocyte colony-stimulating factor (G-CSF), and GM-CSF [283-285]. Not surprisingly, early clinical studies showed that PTP1B levels are up-regulated in cell lines from chronic myelogenous leukemia patients [286, 287]. Therefore, to further explore the potential role that PTP1B might play in regulating myeloid cell development, several groups began examining this lineage in the PTP1B mouse model. The macrophages of PTP1B null mice displayed an increased inflammatory phenotype both *in vitro* and *in vivo*, as became evident by the expansion of the CD80+Ly6G−CD11b+ population in the spleen and an enhanced sensitivity to the endotoxin LPS [282]. Subsequent studies aimed at exploring the potential functional redundancy of the closely related TC-PTP showed that TC-PTP null mice, unlike the PTP1B null mice, display severe immunosuppression, progressive systemic inflammation and die within several weeks of birth [288]. The double mutant mice were used to study inflammation and myeloid cell regulation, and revealed that macrophages isolated from TC-PTP heterozygous and PTP1B null animals are highly sensitive to IFN-γ, as demonstrated by increased phosphorylation of STAT1, these mice also exhibited increased systemic inflammation. Mice heterozygous for both TC-PTP and PTP1B developed normally, suggesting that PTP1B and TC-PTP play nonredundant roles

in macrophage development and IFN-γ signalling [289]. PTP1B is able to negatively regulate cytokine and TLR signalling by decreasing TNF-α, Il-6 and IFN-β production by macrophages stimulated with LPS. This is accomplished through inhibition of MyD88 and TRIF-dependant production of proinflammatory cytokines and type I IFN. [251]. More recently, PTP1B was shown to interact with a myeloid lineage-specific protein PTPIP51 which can influence the MAPK signalling pathway through Raf-1 and 14-3-3β to control proliferation of acute myeloid leukemia cells, as well as play a role in the pathogenesis of glioblastoma multiforme [290-292]. These studies reveal complex roles for PTP1B in the regulation of myeloid cell fate and signalling that are fundamentally distinct from those of TC-PTP. While the results described here begin to paint a picture of how PTP1B can influence signalling in these cells, the final picture is still far from a complete.

2.12.2 B and T lymphocytes.

By crossing PTP1B null mice with the established P53 tumor model, our group observed a higher incidence of B cell lymphomas compared to WT and heterozygous littermates (which mostly succumb T cell lineage lymphomas). Moreover, the bone marrow of PTP1B KO mice presented an increased number of immature $IgM^{-}IgD^{-}$ B cells, thereby implicating PTP1B in the regulation of B cell development. We also observed increased numbers of immature B cells in the peripheral blood and lymph nodes, which can be attributed in part to the observed decrease in the amount of apoptosis but may also be caused by the increased concentrations of circulating cytokines that favor B cell proliferation [266]. Importantly, despite these significant changes in B cell development, there were no differences in the numbers of common lymphoid progenitors (CLPs) between WT and PTP1B knockout mice suggesting that any regulatory roles of PTP1B in these cells is downstream of the CLP. TC-PTP was shown to inactivate the Src family kinases (SFKs) Lck and Fyn, and regulate T cell receptor signalling [293]. Earlier reports revealed a role for TC-PTP and SFKs in the regulation of TNF-induced MAPK signalling [294]. PTP1B has an established role in the regulation of SFKs also by activation through

CSK [295], or inhibition through p62DOK [296]. It is therefore possible that PTP1B and TC-PTP play opposing roles in TNF activated and SFK mediated ERK1/2 signalling leading to altered TCR functioning.

PTP1B is likely to contribute to the downregulation of the inflammatory process especially considering the interplay between obesity and chronic systemic inflammation. The finding that PTP1B expression was controlled by inflammation in mice lent increasing support to the above hypothesis [180]. The authors showed overexpression of PTP1B is concomitant with the increased expression of adipose tissue macrophage markers CD68 and TNF-α, of which TNF-α induces the expression of PTP1B through transcriptional activation by NF-κB. More recently, two studies examined the phenotype of the PTP1B KO both in allergic and obesity-induced inflammation in mice. In the first study, deletion of PTP1B elevated ovalbumin (OVA)-induced lung inflammation as evidenced by altered leukocyte recruitment compared to WT littermates. This recruitment occurs rapidly and early with increases in lung expression of Th2 cytokines and chemokines in KO vs WT mice. Specifically, PTP1B null mice had significant OVA-dependent increases in the chemokines CCL11, CCL24, and MCP-1 and in cytokines IL-13, IL-33, IL-4, IL-5, IL-12, and IFN-γ in the lung tissue. These results show that the lack of PTP1B aggravates allergen-induced inflammation through early recruitment of leukocytes in particular eosinophil progenitors, accompanied by uncontrolled production of proinflammatory molecules [297]. In the second study, PTP1B null mice were protected against inflammation and hypoxia in white adipose tissue in an age related model of obesity. Knockout mice had lower levels of the pro-inflammatory markers CD68, CD11c and TNFα compared to wild type obese mice [298]. These results highlight the important roles that PTP1B plays in regulating inflammation and the consequences it can have on immune signalling as well as metabolism and allergies.

2.12.3 PTP1B in JAK-STAT signalling.

The precise regulation of ligand-dependent and independent cytokine signalling is of paramount importance for maintaining proper immune function. Specific cytokines signal across the cell membrane through the receptor-mediated activation of JAKs, which in turn phosphorylate the membrane receptor, leading to the recruitment and binding of STAT proteins via their SH2 domains. STATs are in turn phosphorylated by the JAKs, followed by STAT dimerization and translocation to the nucleus where they bind specific DNA sequences to modulate target gene expression (Movie 2) [299]. Phosphatases can negatively regulate the JAK-STAT pathway by dephosphorylating either the receptor-associated JAKs or the downstream STATs (**Figure 2.4**). It is therefore tempting to propose that PTP1B also plays an active role in the fine-tuning of these intracellular signalling pathways [300]. It has been known for quite some time that PTP1B specifically dephosphorylates JAK2 and TYK2, but not JAK1, in response to IFNs α and γ [204]. This novel finding was subsequently confirmed by several other laboratories. [205, 225, 226]. Most of these studies, however, were conducted in a metabolic context, and contradicting reports questioned which STATs might also be dephosphorylated by PTP1B. Using the hormone Prolactin Johnson et al. showed increased phosphorylation of JAK2, and STAT5 upon PTP1B knockdown [301]. In fact, STAT3 and STAT5 are major immune regulators and can affect apoptosis, proliferation and mammary gland development [302]. Despite the long-established role for PTP1B in JAK-STAT signalling, it is only recently that we are beginning to understand the specific roles of PTP1B on the JAK-STAT pathways that operate in the immune system [274]. PTP1B has also been found to regulate STAT6 signalling by dephosphorylation upon IL-4 stimulation [281]. IL-4 is a key anti-inflammatory cytokine responsible for the regulation of B cells during an immune response through the activation of STAT6 target genes [303]. IL-4 was shown to induce PTP1B mRNA expression, which acts in an autocrine feedback loop to suppress IL-4 induced STAT6 signalling. Interestingly, PTP1B was also found to be elevated in a subset of B cell lymphomas, further consolidating the link between PTP1B and the regulation of B cells [281]. A more comprehensive

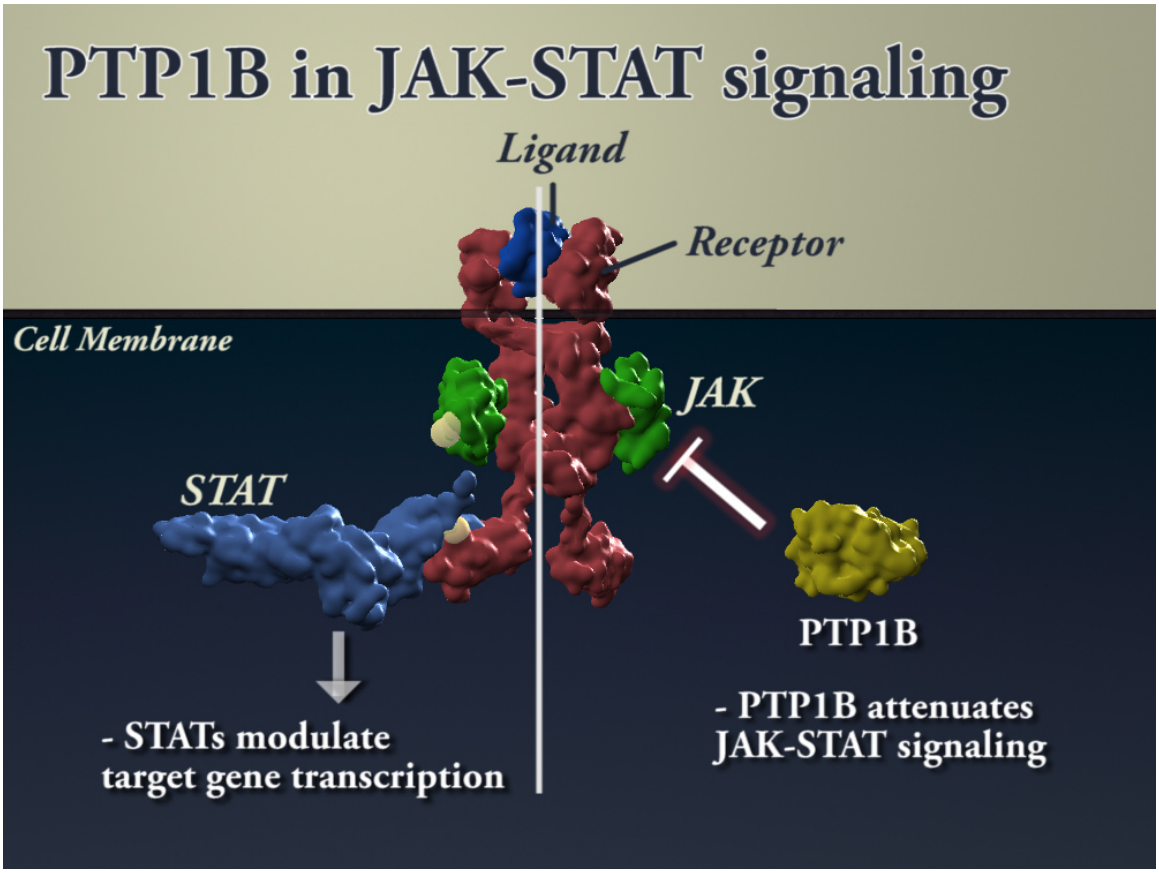

Figure 2.4 Role of PTP1B in JAK-STAT signalling. Ligand binding induces receptor dimerization which in turn activates JAKs. Activated JAKs phosphorylate the membrane bound receptor leading to the recruitment of the STAT transcription factors. PTP1B can then act to dephosphorylate either the receptor-associated JAKs, or the STATs, leading to the attenuation of JAK-STAT signalling.

understanding of the roles that PTP1B plays in the immune system, tissue-specific and inducible mouse models will be indispensable for dissecting its cell type-specific functions and substrates.

2.13 Future perspectives and PTP1B inhibitors.

Since its identification in 1989 over 250 manuscripts have been published on the development of PTP1B small molecule inhibitors. However, as many recent reviews have noted [150, 304], in spite of exemplary biological justifications and a remarkable full KO phenotype that presents minimal side effects, no small molecule inhibitors of PTP1B have yet entered clinical trials. As we summarize in this review, PTP1B remains an outstanding pharmacological target in diabetes, obesity and in some cancers as well. The systematic screening of millions of small molecules with unsuccessful outcomes to date should indicate that the challenge of developing inhibitors against PTP1B, as well as other PTPs will require original screens with innovative chemistry. Already several novel approaches to identify allosteric inhibitors of PTP family members have recently been published. Jeff Mackeigan's laboratory and recent reports by Nunzio Bottini are opening novel approaches [305-307]. Aptamers [308], and novel developments such as intrabodies [201], should also increase our chances to discover modulators of PTP1B, as well as for other members of the large family of PTPs.

In our recent review of the identified roles of PTPs as tumor supressors and oncogenes, we reported that about 30 PTPs had been characterized at different levels as tumor suppressors, and a similar number as oncogenes [139]. Although we can envision the many applications of selectively inhibiting the oncogenic PTPs, would it be useful and even possible to activate specific PTPs in some diseases? One can anticipate that such a strategy could produce extremely active PTPs that might target the aberrantly expressed RTKs in cancer. Neither should we forget that the targeting of intramolecular inhibitory sequences, blocking the dimerization of receptor PTPs (which should activate the monomer), as well as reversing epigenetically silenced PTPs, may be potential

avenues too. Yet for PTP1B, an enzyme that may act in opposite ways depending of the cellular context, this controversy may be extremely difficult to resolve.

Interestingly the reported roles of several PTPs (including PTP1B) in the differentiation of stem cells could lead to important applications. One may see how, for instance, specific stem cells may be treated *ex vivo* (even with suboptimal PTP inhibitors) in order to promote their differentiation into particular and useful cell types, which could then be used in cellular therapies. In a recent review Pike and Tremblay described the various PTPs involved in the differentiation of T cell lineages, demonstrating the inherent complexities and synergies that occur amongst many members of the PTP family [309]. Moreover in a recent paper by Bourdeau *et al*, the inhibition of TC-PTP was shown to lead to an increase in the numbers of several types of hematopietic stem cells (HSCs) . Large numbers of HSCs cultured *ex vivo* could then be used in a broad array of cellular applications, such as the amplification of cord blood cells and HSCs, and that of endothelial progenitor cells for repairing ischemia damage.

2.14 Conclusions

Among all members of the complex and ancient PTP gene family, PTP1B embodies the paradigm of a relatively simple enzyme with the ability to dephosphorylate many different substrates, thus representing a very complex therapeutic target. As illustrated with the mouse knockout and the non-specific inhibitor vanadium, PTP1B appears to be one of the most suitable targets for the treatment of type II diabetes and obesity. When PTP1B is overexpressed, it is clear that it promotes metabolic diseases and therefore it will remain at the center of many pharmaceutical and academic efforts to develop specific inhibitors to treat these conditions. For these efforts to succeed, more original and out-of–the–box thinking will be necessary to open the untapped potential of PTPs in medical applications.

2.15 Acknowledgements

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2.16 Declaration of interest

The authors report no conflict of interest. Funding was provided (grant MOP-62887) to M.L.T by the Canadian Institutes of Health Research M.L.T is a James McGill Professor and holder of the Jeanne and Jean-Louis Lévesque Chair in Cancer Research. M.F is a recipient of a Doctoral Research Award from Canadian Institutes of Health Research

2.17 Overview and rational for thesis

To date, PTP1B has been well established as a critical regulator of both metabolic and cancer signalling. PTP1B was shown to exert both tumor promoting and tumor suppressing roles depending on the cellular substrate it targets. Despite the fact that some of the first identified substrates of PTP1B were critical immunomodulators of the JAK-STAT family, very little research has uncovered roles for PTP1B in modulating the immune system. Recently our laboratory and others have begun to explore the functions of PTP1B in a variety of immune cell lineages and have uncovered key functions in B cells, macrophages and the inflammatory response. These findings support the concept that PTP1B may play a vital function in regulating the immune system.

An impaired immune response is a hallmark of cancer and represents a distinct challenge to overcome in the development of effective anti-cancer therapies. Tyrosine phosphorylation is an important and reversible intracellular post-translational modification implicated in controlling a variety of immune cell functions. Aberrant phosphorylation signals can result in immune suppression and the development and progression of a number of malignancies.

Immunotherapies represent a promising approach in the treatment of a variety of cancers and proper dendritic cell signalling is critical as they have the potential to effect potent and long lasting anti tumor immunity. Dendritic cell activity is governed to a large extent by the activation of genes regulated in a phosphorylation dependent fashion. We therefore aimed to answer how PTP1B mediates the immune response to cancer, and if we could enhance immune signalling and cancer immunotherapies by modulating PTP1B activity.

CHAPTER 3: PTP1B DEFICIENCY LEADS TO EHNHANCED LYMPHOMA PROGRESSION DUE TO COMPRIMISED DENDRITIC CELL FUNCTION

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3.1 Preface to the manuscript

PTP1B substrates have been implicated in the control of metabolism, cancer and immune cell signalling. However, the majority of work to date has focused primarily within the contexts of metabolism and cancer. While contributing to these important bodies of work, our lab has begun to explain how PTP1B regulates the differentiation and function of a variety of immune cells, which play vital roles in controlling the body's response to cancer. We therefore sought to evaluate how the immune system of PTP1B deficient mice responds to tumor challenge and determine which immune cell types were affected by a loss of PTP1B.

3.2 Abstract

PTP1B is a critical modulator in metabolic signalling, cancer and immunity. Although some of the first identified PTP1B substrates belong to the JAK-STAT immunomodulatory family, our understanding of the role that PTP1B plays in the immune system remains largely undefined. Here we show using a syngenic Eμ-myc model of B cell lymphoma that PTP1B null mice have an impaired immune response to tumor challenge leading to decreased survival and increased tumor cell proliferation. Mice lacking PTP1B displayed increased numbers of immature dendritic cells as well as increased myeloid derived suppressor cells in tumor infiltrates. Moreover, dendritic cells (DCs) lacking PTP1B are maturation compromised and have less potent antigen presentation capacity based on decreases in cytokine secretion, surface MHC class II production and a reduction in $CD4^+$ T cell activation. PTP1B null DCs display hyperactivation of STAT3 and STAT5 through increased tyrosine phosphorylation and have decreased levels of IL-12 secretion, which contributed to inhibition of DC maturation and activation. To examine specifically what role PTP1B plays in DCs the PTPN1 gene was targeted for deletion with a CD11c-cre transgene. DC specific PTP1B knockout (KO) mice displayed similar overall survival to the whole body KO model. Our results provide novel insights into the role that PTP1B plays in DC development and

indicate that PTP1B deletion negatively affects DC maturation thus demonstrating that PTP1B plays a positive role in maintaining overall immune tumor surveillance.

3.3 Introduction

Based for the most part on the identification of a broad number of substrates, PTP1B has been recognized as a key modulator of insulin receptor and leptin signalling in metabolic syndromes [148, 149]. PTP1B has also been reported to directly dephosphorylate other growth factor receptors and cytoplasmic kinases involved in signalling pathways targeted by many oncogenic processes such as c-src kinase and p120RASGAP that influence metabolic signalling, as well as cancer initiation and progression [266, 271]. Notably, our group and others have shown that PTP1B can play a tumor suppressive role in a p53 KO model of T cell lymphoma as well as B cell and Hodgkin's lymphomas [266, 310]. Despite these reports highlighting a tumor suppressor function for PTP1B, other cellular and animal findings identified PTP1B as presenting oncogenic functions in breast, prostate, and most recently gastric and lung cancers [181, 266, 269, 310, 311]. This dual nature of PTP1B has been well documented and is likely due in part to the diversity of substrates it can actively dephosphorylate in a given cellular context [140, 262, 312]. For the most part, these studies have only considered cell autonomous roles for PTP1B and have neglected the potential systemic functions that may be altered in a PTP1B deficient state. The paradoxical role of the immune response to cancer is well documented [3, 313-316] however the role that PTP1B may play in mediating the anti-tumor immune response is only now beginning to be explored. Because of its broad range expression, PTP1B has been found to regulate myeloid cell development and exerts control over macrophage function and the inflammatory response [180, 251, 289, 297, 317, 318]. We therefore aimed at uncovering how PTP1B regulates the immune response to tumor challenge and examine if the expression levels of PTP1B may influence tumor development.

Initial investigations by our group into the role of PTP1B in cancer led to the discovery that mice doubly deficient for p53 and PTP1B were prone to the development of B cell lymphomas. This observation came as a surprise owing to the fact that p53 deficient animals generally present a greater number of T cell lymphomas than B cell lymphomas. This switch in lymphoma cell type specificity was attributed to a preferential block in B cell development at the pre-B cell stage favoring the accumulation of immature B cells in the bone marrow and lymph nodes in the double knockout mice [266]. These results represented some of the first sets of evidence that PTP1B had a significant effect on development of the immune system, with discernable systemic effects on tumor progression. Despite the early discoveries that critical immunomodulators such as TYK2, JAK2, STAT3 and STAT6 are PTP1B substrates [204, 281, 319], a defined role for PTP1B in the immune system has remained elusive until recently. Our lab and others have demonstrated that PTP1B null mice exhibit chronic low-grade inflammation partially owing to deficiencies in myeloid cell development [289, 297]. Specifically, PTP1B deficient macrophages displayed heightened inflammatory phenotypes characterized by expansion of $CD80^{+}Ly6G^{-}CD11b^{+}$ population in the spleen and an enhanced sensitivity to the endotoxin lipopolysaccharide (LPS). More recently we reported that PTP1B null macrophages had enhanced expression of anti-inflammatory genes as well as enhanced IL-10 dependent STAT3 phosphorylation [318]. Taken together, these findings highlight an emerging role for PTP1B as an important factor in the regulation of monocyte derived immune cells.

Myeloid precursors give rise to several immune cell types including monocytes, macrophages, granulocytes and myeloid DCs. Myeloid development depends on JAK2- and TYK2-mediated signalling both of which have been shown to be directly dephosphorylated by PTP1B. PTP1B KO mice have been characterized as having an abnormal monocyte/granulocyte ratio due to an increased number of colony stimulating factor (CSF-1)-responsive cells and an increased sensitivity to receptor-ligand interaction [282]. PT1PB was further shown to regulate the activation state of

monocyte/macrophage lineage cells owing to the expression of the activation marker CD80. Based on these findings we hypothesized that PTP1B may influence the activation and function of other monocytic lineage cells due to its ability to dephosphorylate the common JAK2 and TYK2 signalling cascades. We therefore sought to investigate the role of PTP1B in the anti-tumor immune response.

We report herein that PTP1B is required for the proper maturation and function of monocyte derived DCs. Using *in vivo* imaging in both whole body and DC specific KO mice we show that mice lacking PTP1B more rapidly succumb to tumors owing to a deficiency in their ability to mount a proper immune response. This altered immune response is due to increased numbers of immature and functionally compromised DCs infiltrating tumors, as well as increased amounts of the immunosuppressive myeloid derived suppressor cells (MDSC). Deletion of PTP1B in DCs caused an increased activation of inhibitory JAK-STAT signalling and a decreased expression of MHC class II and the costimulatory molecule CD40 on DC cell surface. Moreover, these PTP1B-deficient antigen-presenting cells (APCs) are unable to properly elicit a $CD4^+$ T cell response. We conclude that deficiency in PTP1B expression in DCs impairs systemic DC maturation, thus preventing proper immune surveillance and hindering tumor cell clearance.

3.4 Results

3.4.1 Deficiency of PTP1B leads to decreased survival and increased tumor cell proliferation in an Eμ-myc lymphoma tumor challenge.

We set out to determine the contribution of PTP1B to the immune response using an Eμ-myc lymphoma tumor model. Previously our lab and others have shown that PTP1B can act as either a tumor suppressor or an oncogene depending on the nature of the phosphotyrosine substrate or tumor model used [262, 266, 267, 269, 310, 312]. Because PTP1B has already been shown to dephosphorylate JAK2 and TYK2 [204], we hypothesized that lack of PTP1B would result in hyperactivation of JAK-STAT signalling.

This in turn would manifest in an overactive immune response upon tumor challenge and lead to an enhanced anti-tumor capability and increased survival. Eμ-myc lymphomas are highly transplantable and can be titrated down to 10 cells/recipient mouse and still induce tumorigenesis with similar pathologies and disease onset times [320]. In order to select the optimal cell number for challenging PTP1B$^{-/-}$ mice, a dose response of low (10^2 cells) to high (10^6 cells) was tested. When C57Bl/6n mice were challenged via tail vein injections with syngeneic 10^6, 10^5, 10^4 or 10^3 Eμ-myc lymphoma cells, no differences were noted in tumor onset between PTP1B$^{-/-}$ and PTP1B$^{+/+}$ animals (**Supplemental Figure 3.8 A-D**). However, at a low initial tumor burden of 10^2 cells, PTP1B$^{-/-}$ mice demonstrated a significant decrease in overall survival (median survival 19 days, n=22) compared to wild type (WT) littermates (median survival 24 days p****<0.0001, n=24) **(Figure 3.1 A**).

Towards monitoring disease progression, we generated a dual reporter (GFP-Luciferase) construct utilizing a Murine Stem Cell Virus (MSCV) -based expression system [321]. This MSCV vector was then used to infect the Eμ-myc lymphoma cells prior to implantation in order to monitor the lymphoma progression in real time. *In vivo* imaging of the tumors over the course of the study revealed that PTP1B$^{-/-}$ mice displayed significantly enhanced lymphoma cell proliferation compared with the WT littermates particularly after day 15 (**Figures 3.1 B-C**). In addition we noted that tumor onset was also delayed in WT mice compared with their PTP1B$^{-/-}$ littermates. Palpable tumors were detected at day 14 vs day 20 for PTP1B$^{+/+}$ vs PTP1B$^{-/-}$ respectively (data not shown), indicating that an immune component responsible for tumor surveillance could be affected in the PTP-deficient mice.

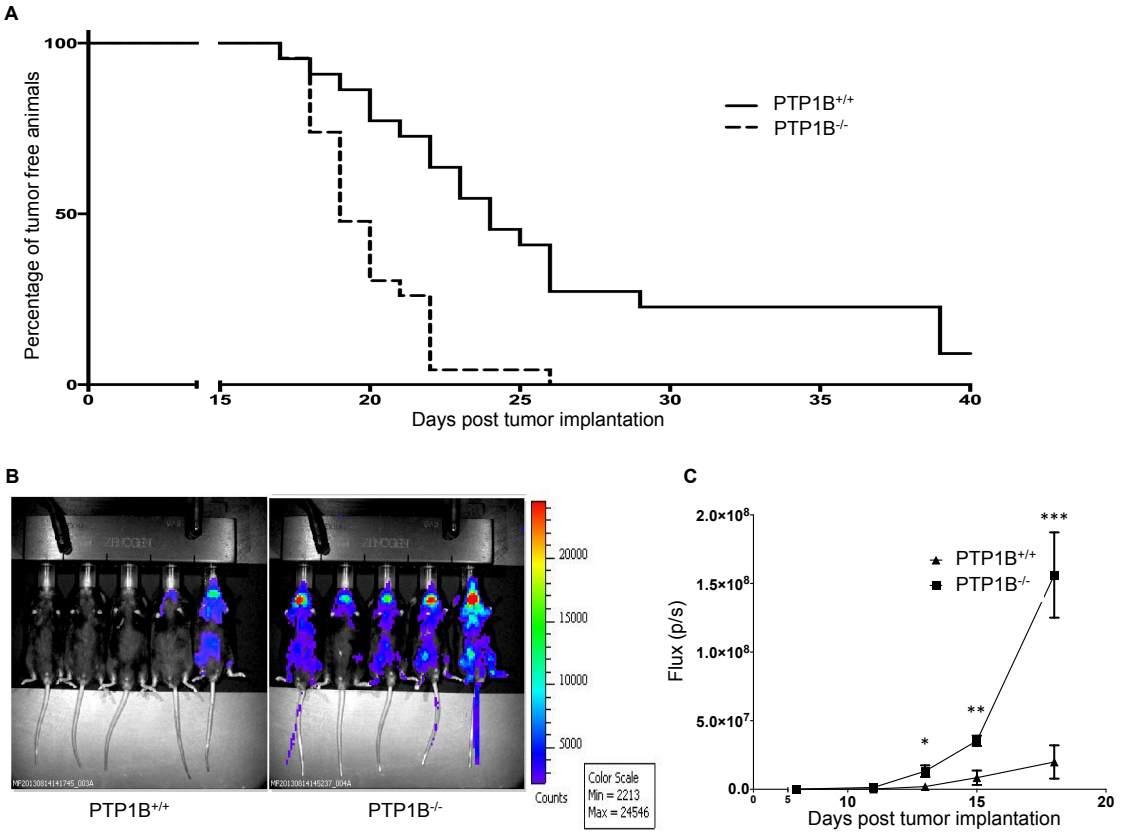


Figure 3.1 PTP1B null mice exhibit decreased survival and enhanced tumorigenesis when challenged with tumors from Eμ-myc mice. B cell lymphomas derived from Eμ-myc mice were infected with a GFP-Luciferase dual reporter vector (material and methods) and 100 tumor cells were injected via tail vein into syngeneic male six to ten week old WT or PTP1B$^{-/-}$ C57bl/6n mice. Mice were monitored for tumor formation and progression via palpation of the inguinal and axillary lymph nodes and *in vivo* bioluminescent imaging. **A**) Kaplan-Meier analysis of survival of tumor bearing mice (n=22) harboring WT or deletion of *ptpn1* gene ****p<0.0001. **B**) Lymphoma progression was monitored by *in vivo* bioluminescence imaging taken at days (8, 11, 13, 15, 18) until saturation of the imager precluded further monitoring. Representative images of differences in tumor dissemination between PTP1B$^{+/+}$ and PTP1B$^{-/-}$ on Day 18 are shown. **C**) Quantification of tumor progression as measured by bioluminescent intensity over the duration of the study. The flux (photons/second) was quantified for each mouse per group and the means were plotted.

3.4.2 PTP1B deletion induces the production of Th1 and Th2 cytokines and chemokines.

In order to explain the phenotype we observed in our PTP1B$^{-/-}$ mice, we hypothesized that PTP1B was acting to tip the immune system balance towards a Th2 based response. The Th2 anti-inflammatory response is associated with increased expression of IL-4 as well as IL-10 and can favor tumor growth [322-324]. In contrast, the Th1 pro-inflammatory cell mediated immune response is characterized by increased IL-12 and interferon gamma (IFN-γ) production, and can promote anti-tumor activity [87, 325]. We investigated cytokine levels of both WT and PTP1B$^{-/-}$ mice using a broad-spectrum serum cytokine array (**Figure 3.2 A**). To our surprise the main Th1 (IL-2, **P=0.005; IL-12 *P=0.02; and IFN-γ *P=0.02) and the majority of Th2 (IL-4 ***P<0.0001; IL-10 *P=0.02; IL-13 *P=0.03) cytokines were elevated in the PTP1B$^{-/-}$ mice (**Figure 3.2 B-D**). These results therefore did not provide any specific insight into which branch of the immune system influenced the tumor onset and growth in our PTP1B null mice. We then examined the main cytokines responsible for promoting B cell proliferation (IL-2 **P=0.005; IL-6 *P=0.03 and IL-7 *P=0.03) and discovered that these were also elevated in PTP1B$^{-/-}$ mice (**Figure 3.2 B-D**). Importantly, cytokines and chemokines were not universally elevated in the PTP1B KO mice. The angiogenic chemokine stromal cell-derived factor 1 (SDF-1) was not found to be different between the two models (**Figure 3.2 D**, SDF-1, p=0.27) and plays an important role in the migration of a number of immune cells [326]. As well, the main cytokine responsible for stimulating the production of granulocytes and monocytes, Granulocyte-macrophage colony-stimulating factor (GM-CSF) was comparable between WT and KO mice (**Figure 3.2 D,** P=0.11). Several other cytokines and chemokines were also not found to be significantly different between WT and PTP1B$^{-/-}$ mice (**Figure 3.2 D**). However, the majority of cytokines and chemokines tested (25/40, P<0.05) were elevated in the PTP1B deficient mice and there appeared to be no discrimination between increases in Th1 vs Th2 cytokines. We therefore observed that the immune system in PTP1B$^{-/-}$ mice appears to be hyper-activated but neither favors a humoral or cell mediated immune response, instead it is indicative of a systemic inflammatory response. Increased cytokine

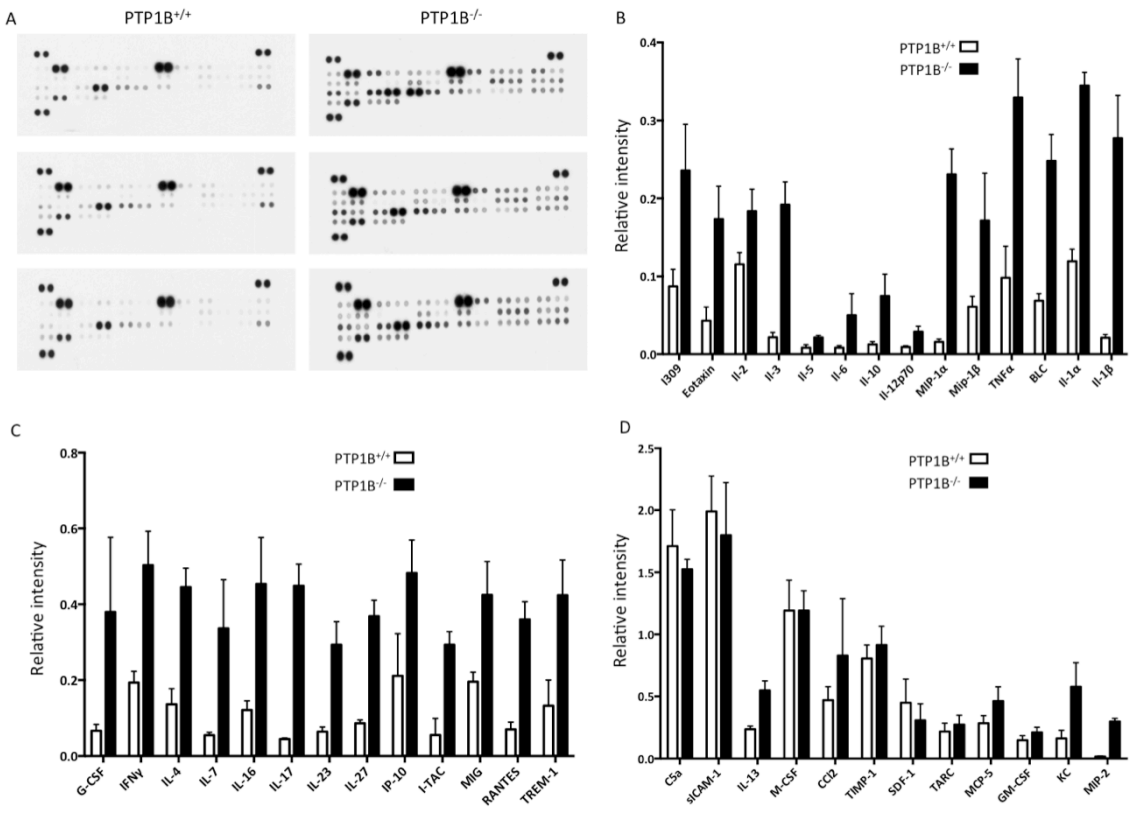


Figure 3.2 Aberrant serum cytokine and chemokine levels of PTP1B null mice. **A**) Cytokine and chemokine protein array blots of male six to ten week old PTP1B$^{-/-}$ and PTP1B$^{+/+}$ littermates. Cytokines and chemokines in mouse sera were detected using the mouse Proteome Profiler antibody array (R&D systems) following the manufacturers protocol. Each panel was conducted in quadruplicate. **B-D**) Quantification of cytokine and chemokine levels of PTP1B$^{-/-}$ (black) and PTP1B$^{+/+}$ (white) mice was done with the ChemiDoc molecular imager. The intensities of each spot were quantified and the means were graphed relative to the mean of the intensities of the control cytokines on the panel.

production has also been observed in the lungs of ovalbumin (OVA) challenged PTP1B$^{-/-}$ mice [297]. Additionally PTP1B$^{-/-}$ mice have been characterized as having chronic low-grade inflammation [289, 297, 327], which has been attributed at least in part to changes in myeloid cell development and activation.

3.4.3 PTP1B affects the adaptive immune response to tumor challenge.

In order to investigate which subset of the immune system was affected upon tumor challenge in the absence of PTP1B, one needs to identify those cells, which were differentially mediating the anti-tumor response. To rigorously answer this fundamental question, we employed an immune-infiltration assay to determine which immune cells were present at the tumor site [328, 329]. No differences were observed in the total number of infiltrating lymphocytes (**Figure 3.3 A**) suggesting that any differences may be reflected in the monocyte populations. Furthermore we noted no differences for the majority of common immune lineage markers for CD4, CD8 natural killer (NK), natural killer T cells (NKT) and macrophages (**Supplemental Figure 3.9 A-D**). We did however notice a slight difference in the number of CD11c^{+} dendritic cells (**Figure 3.3 B**) infiltrating the tumor in the PTP1B$^{-/-}$ mice when compared with WT littermates. Dendritic cells are the professional APCs in the body and are responsible for coordinating B and T cell function and antitumor response [330, 331]. To determine how increased numbers of DCs present at the tumor site would affect the immune response we looked at expression levels of the major histocompatibility complex (MHC) on the DCs cell surface. We noted that the increased numbers of DCs present at the tumor site of PTP1B$^{-/-}$ mice expressed lower levels of class II (**Figure 3.3 C**), and a trend in decreasing MHC I expression (p=0.06) (**Figure 3.3 D**) indicative of immature tolerogenic DCs [332, 333]. Immature DCs have an increased capacity to uptake antigen, but are not able to properly present antigen to CD4^{+} or CD8^{+} T cells, and therefore are unable to coordinate the anti-tumor immune response. We also noted increased numbers of CD11b^{+} Ly6G^{+} myeloid derived suppressor cells (MDSC) (**Figure 3.3 E**) In line with our results, ablation of PTP1B was also shown to dramatically increase the number of

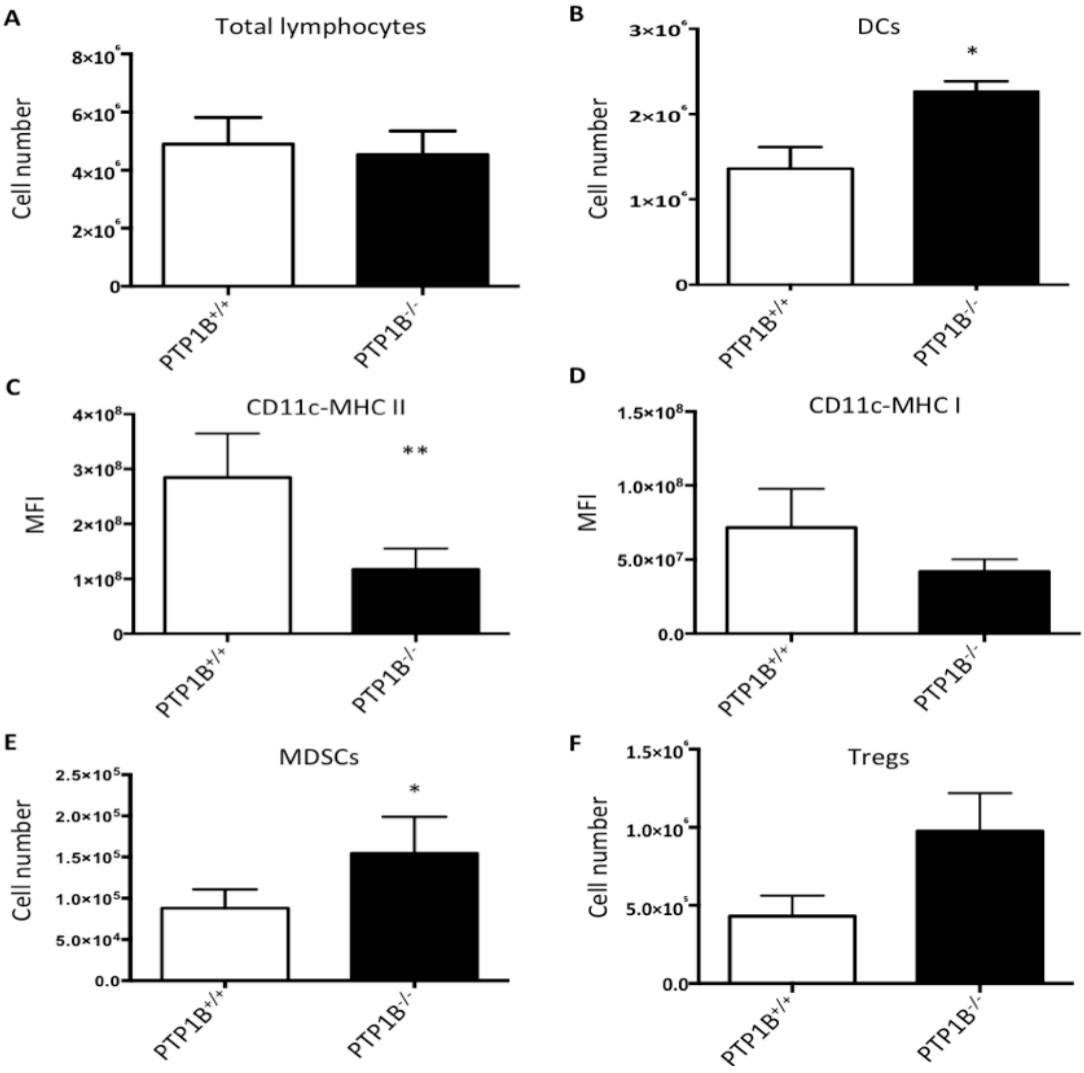


Figure 3.3 Increased recruitment of immature DCs and suppressive immune cells present in tumor grafts of PTP1B$^{-/-}$ mice. WT and PTP1B null mice received subcutaneous injections of 10^6 Eμ-myc B cell lymphomas mixed in Matrigel. Implants were then surgically removed after 7 days, dissolved to single cell suspensions, and analyzed by flow cytometry for the presence of **A**) total lymphocytes, **B**) dendritic cells, **C**) myeloid derived suppressor cells, and **D**) regulatory T cells depicted in histograms of mean cell number per implant ± SEM (*n* = 4 per group). **C-D**) MHC I and MHC II expression was determined as MFI on the population of CD11c positive cells present in the tumor grafts. Results are representative of at least two independent experiments. For Mann-Whitney tests, *, $P < 0.05$; **, $P < 0.005$.

MDSCs in a mouse model of colitis [334]. There is considerable evidence to support an immune suppressive function for immature DCs either by the induction of anergy, deletion of antigen reactive T cells or stimulation of suppressive regulatory T cells (Tregs) [59, 335-337]. In accordance, we observed trending though not statistically significant increases in the numbers of infiltrating Tregs in our tumor grafts in PTP1B$^{-/-}$ mice in two out of three experiments performed (**Figure 3.3 F**). These results demonstrate that there are increased numbers of suppressive immune cells present within our tumor model explaining the observed increased tumor proliferation and decreased survival observed in the PTP1B$^{-/-}$ mouse model.

3.4.4 PTP1B null mice have an immature DC phenotype

The transition from an immature to a mature DCs is characterized by dramatic phenotypic changes such as up regulation of MHC class I and II molecules, costimulatory molecules, T-cell adhesion markers, chemokine receptors and cytokine production [49, 338, 339]. To specifically assess the impact of PTP1B on the phenotype of bone marrow derived dendritic cells (BMDCs) we derived BMDCs from the femur and tibia PTP1B$^{-/-}$ and PTP1B$^{+/+}$ mice. BMDCs derived from PTP1B$^{-/-}$ mice displayed a more immature phenotype than those derived from WT littermates. Immature DCs are found in many cancers and can produce tolerogenic effects and support tumor cell proliferation by expanding the pool of Tregs and other suppressive factors [45, 340]. Specifically, PTP1B$^{-/-}$ BMDCs displayed no difference in MHC I expression (**Figure 3.4 A**) but showed significantly decreased MHC class II expression (**Figure 3.4 B**)**.** We also noted decreased expression of the costimulatory molecule CD40 (**Figure 3.4 C**) while no differences in CD86 were noted **(Figure 3.4 D)**. Furthermore we observed decreased expression of the antigen binding and processing receptor CD205 [341] (**Figure 3.4 E**) in PTP1B null BMDCs upon exposure to LPS as a maturation stimulus compared with WT DCs. The significant decrease of MHC class II and co-stimulatory molecule expression observed in PTP1B KO BMDCs is characteristic of immature tolerogenic DCs. As controls, we also examined MHC class I, class II, and costimulatory molecule expression on DCs deficient for other

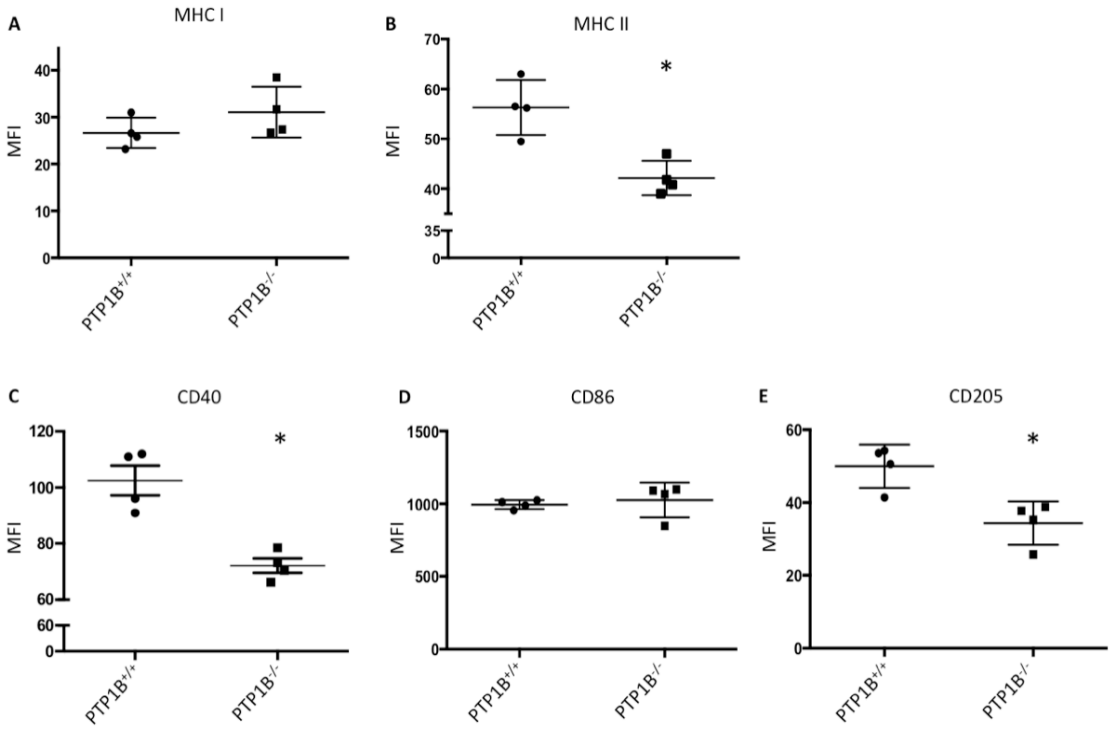


Figure 3.4 PTP1B null BMDCs have decreased MHC II and costimulatory marker expression. A-E) Mouse bone marrow monocytes were surgically extracted from femurs and tibias. Monocyte enrichment using CD11b positive selection was performed and $15x10^6$ monocytes were cultured for 7 days before maturation was induced with LPS to obtain mature BMDCs. Cells were then stained for CD11c and the indicated markers with specific fluorescence conjugated antibodies and their expression upon maturation was determined by flow cytometry. The results are representative of three independent experiments (except for CD40 and CD205 which was performed twice with n=4). Significant differences are denoted by asterisks *, $P < 0.05$ (data are shown as the mean, n=4).

non-related phosphatases including PTPRS, PTPRD and PTPRF. No differences were observed between PTP defective DCs and their respective WT DC controls (data not shown).

Because of the tumorigenic phenotype we observed in the $PTP1B^{-/-}$ mice we hypothesized that PTP1B null DCs were unable to properly direct a Th-1 immune response. $CD4^+$ T helper cells are able to polarize into either a Th1 or a Th2 directed immune response based on their stimulatory environment. Th-1 responses are typically more effective in eliciting an anti-tumor response through the production of IFN-γ, IL-12 and the enhancement of the anti-tumor cytotoxic T-lymphocyte responses [342, 343]. We assessed the ability of PTP1B deficient BMDCs to produce Th1-directing cytokines, as well as inflammatory and suppressive cytokines. Mature $PTP1B^{-/-}$ DCs produced significantly lower levels of the main Th1-promoting cytokine IL-12 (**Figure 3.5 A**). Surprisingly no differences were observed in the immunosuppressive cytokines IL-10 and IL-6 or transforming growth factor beta (TGF-β) (**Figure 3.5 B-D**). These results suggest that PTP1B expression is required for proper DC maturation, activation and in the production of Th1 polarizing cytokines.

DCs are endowed with the ability to present antigen and activate T cell responses. We utilized co-culture assays in order to determine the antigen-presentation capacity of PTP1B deficient BMDCs, measuring the T cell-derived cytokine IL-2 production as readout. We analyzed the ability of mature $PTP1B^{+/+}$ and $PTP1B^{-/-}$ deficient BMDCs pulsed with chicken ovalbumin (OVA) peptide to activate $CD8^+$ or $CD4^+$ T cells derived from OT-I or II transgenic mice, respectively. T cell receptors (TCRs) from OT-I transgenic mice recognize OVA residues 257-264 in the context of MHC I [344], whereas the OT-II TCR is specific for 323-339 residues in the context of MHC II [345]. As predicted OVA-pulsed $PTP1B^{-/-}$ DCs displayed a significantly lower capacity for OT-II $CD4^+$ T cell activation (**Figure 3.5 E**), which correlates with the previously observed decrease in MHC class II expression (**Figure 3.4**). No differences were observed in the ability of OVA

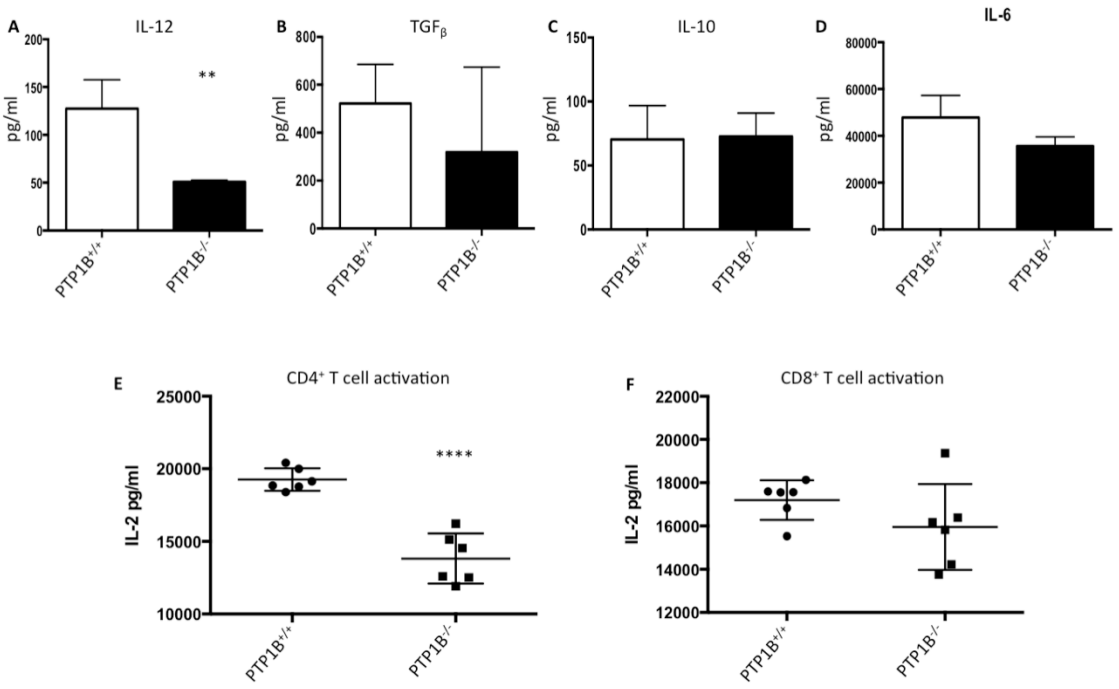


Figure 3.5 Decreased cytokine production and antigen presentation capacity upon loss of PTP1B. BMDCs were derived as in Figure 4 from PTP1B$^{+/+}$ and PTP1B$^{-/-}$ mice. Maturation was induced with LPS (**A-D**) or LPS + OVA (**E-F**) for 24 hours at which point, supernatants were collected and frozen until use. **A-D**) Supernatants were then tested for the presence of IL-12, TGFβ, IL-10 and IL-6 cytokine production as indicated. **E-F**) LPS + OVA stimulated DCs were co-cultured with OT-1 (**E**) or OT-II (**F**) T cells and the amount of IL-2 was determined. Significant differences are denoted by asterisks as determined by Mann-Whitney tests, **, $P < 0.005$, ****, $P<0.00005$ (data are shown as the mean of two independent experiments each with n=3-6).

pulsed PTP1B$^{+/+}$ BMDCs to activate OT-I CD8^{+} T cells (**Figure 3.5 F**). These data suggest that PTP1B expression is essential for proper DC maturation and antigen presentation and can subsequently negatively affect the ability of DCs to induce CD4^{+} T cell activation.

3.4.5 PTP1B deletion results in hyperactivation of JAK-STAT signalling in mature BMDCs

Previous studies have established JAK2 and TYK2 as direct substrates of PTP1B [204]. JAK2 and TYK2 signal through STAT3 and STAT5 in DCs and their activation has been shown to negatively regulate DC maturation through the downregulation of MHC class II expression as well as inhibit the production of key cytokines [84, 90, 327]. We therefore hypothesized that PTP1B deficient BMDCs would have higher activation of STAT3 and STAT5 resulting in decreased MHC class II expression (**Figure 3.4 B**) as measured by the amount of phosphorylation of their activation sites. Our analysis of the JAK-STAT pathways (**Figures 3.6 A-C**) reveals increased phosphorylation and activation levels of JAK2, STAT3 and STAT5 in the mature PTP1B$^{-/-}$ BMDCs. In accordance with previous studies, JAK2 and TYK2 activation in DCs is essential for the production of Th1-promoting cytokine IL-12 and therefore the induction of antigen-specific Th1-cell differentiation and proper DC maturation [84, 346]. Deletion of PTP1B results in a marked activation of JAK2 (**Figure 3.6 A-B**) resulting in hyperactivation of downstream transcription factors STAT3 and STAT5 (**Figure 3.6 C**), which have been show to negatively regulate DC maturation through downregulation of MHC class II expression. These data suggest that modulation of PTP1B expression significantly affects important DC receptor signalling resulting in altered DC maturation.

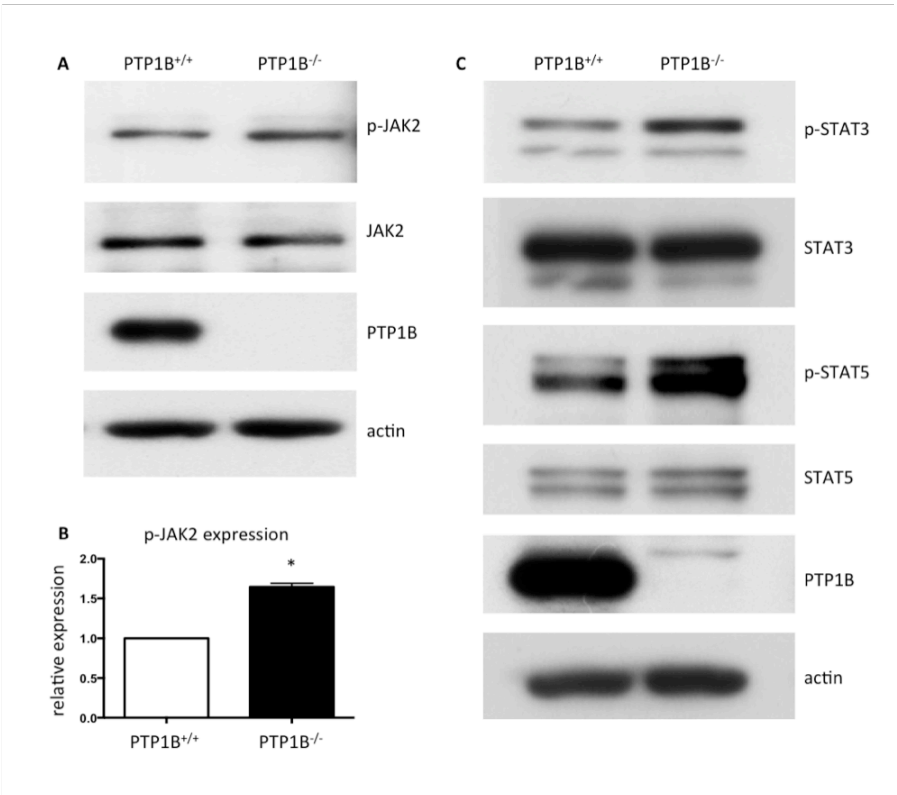


Figure 3.6 Loss of PTP1B results in hyperactivation of JAK-STAT signalling in BMDCs. A) BMDCs were generated from PTP1B$^{+/+}$ and PTP1B$^{-/-}$ mice as in Figure 4. Twenty-four hours after LPS stimulation cells were harvested, lysed and subjected to SDS-PAGE. Lysates were immunoblotted for activation of JAK2 using phospho-specific antibodies for Tyr-1008/1008, and reprobed for total JAK2, PTP1B and actin **B**) The blots were scanned, and bands corresponding to phosphorylated JAK2 were quantified using National Institutes of Health ImageJ software. The results are the mean of phosphorylated JAK2 (from three independent experiments) normalized to levels of actin. **C**) BMDC lysates were probed for total as well as phospho-specific STAT3 (Tyr-705) and STAT5 (Tyr-694). Immunoblots were subsequently striped and probed for PTP1B to confirm expression and actin as loading control.

3.4.6 Decreased survival of PTP1B$^{-/-}$ mice challenged with Myc-driven lymphomas is due to lack of PTP1B in DCs.

To validate the hypothesis that PTP1B deficient DCs were responsible for the survival defect observed in mice challenged with Eμ-myc lymphomas, we generated mice carrying a DC-specific deletion of PTP1B as described in the Methods section. The efficiency and specificity of deletion in the *Ptpn1*$^{fl/fl}$-CD11cCre mice was assessed by Western blot and found to be specific for mature BMDCs and did not affect the closely related phosphatase TC-PTP (**Figure 3.7 A and Supplemental Figure 3.10**).

Ptpn1$^{fl/fl}$-CD11cCre mice were challenged with Eμ-myc lymphomas in the same fashion as the full body PTP1B KO mice (**Figure 3.1**). In agreement with our hypothesis that PTP1B deficient DCs are less able to mount an effective immune response to tumor challenge, *Ptpn1*$^{fl/fl}$-CD11cCre mice displayed an overall decrease in survival (median survival 18 vs 28 days) compared with either *Ptpn1*$^{wt/wt}$-CD11cCre^{+} or *Ptpn1*$^{fl/fl}$-CD11c-Cre^{-} controls (**Figure 3.7 B**). In addition, tumor proliferation was monitored *in vivo* and in accordance with results obtained above, tumor proliferation was more rapid in *Ptpn1*$^{fl/fl}$-CD11cCre compared with controls (**Figures 3.7 C-D**). These results confirm that PTP1B is essential in the generation of fully mature and functional DCs, and is required to properly mount an immune response upon tumor challenge.

Thus, PTP1B is necessary for proper DC maturation and priming of Th1 T cell responses. Lack of PTP1B is associated with decreased MHC II and co-stimulatory molecule expression, decreased IL-12 production and aberrant JAK-STAT signalling in BMDCs resulting in ineffective immune responses upon a lymphoma tumor challenge.

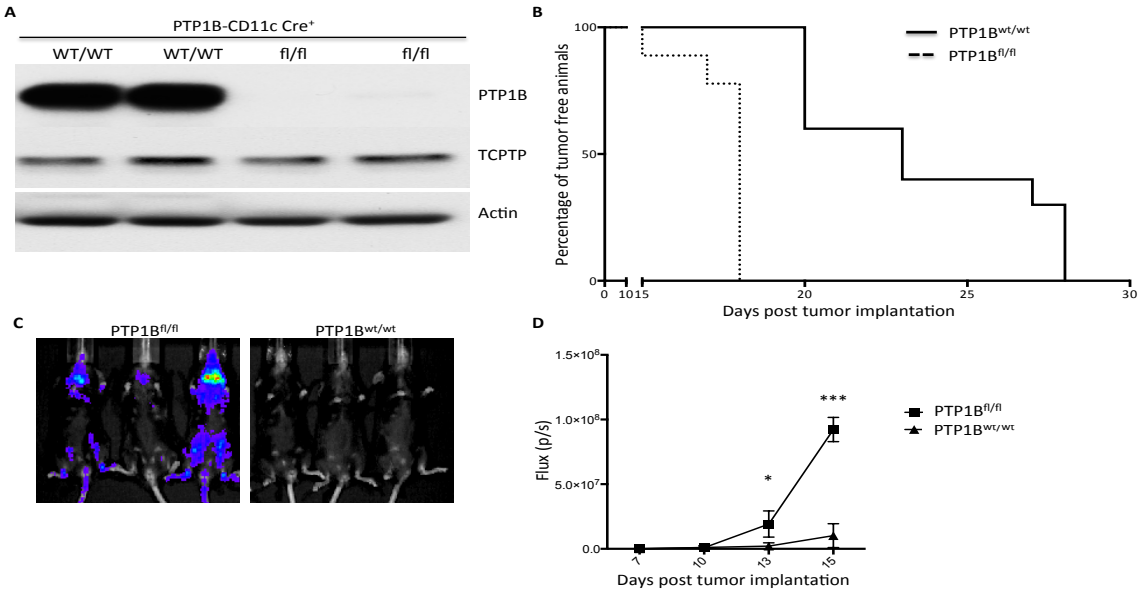
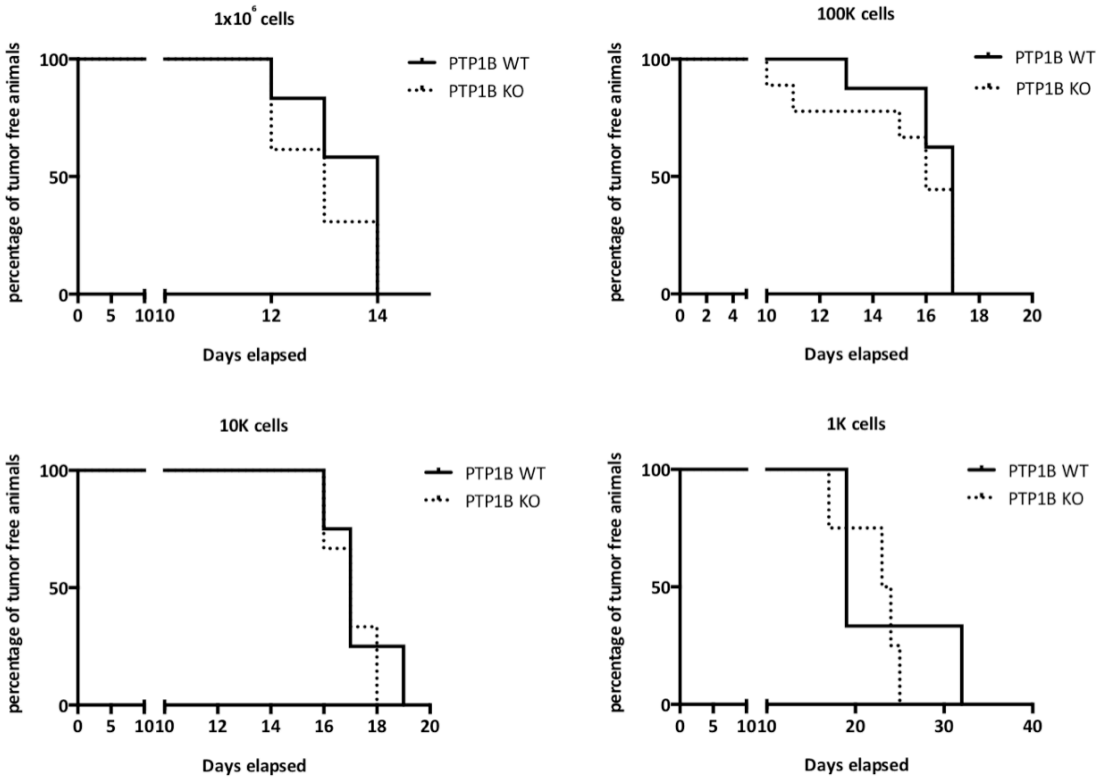
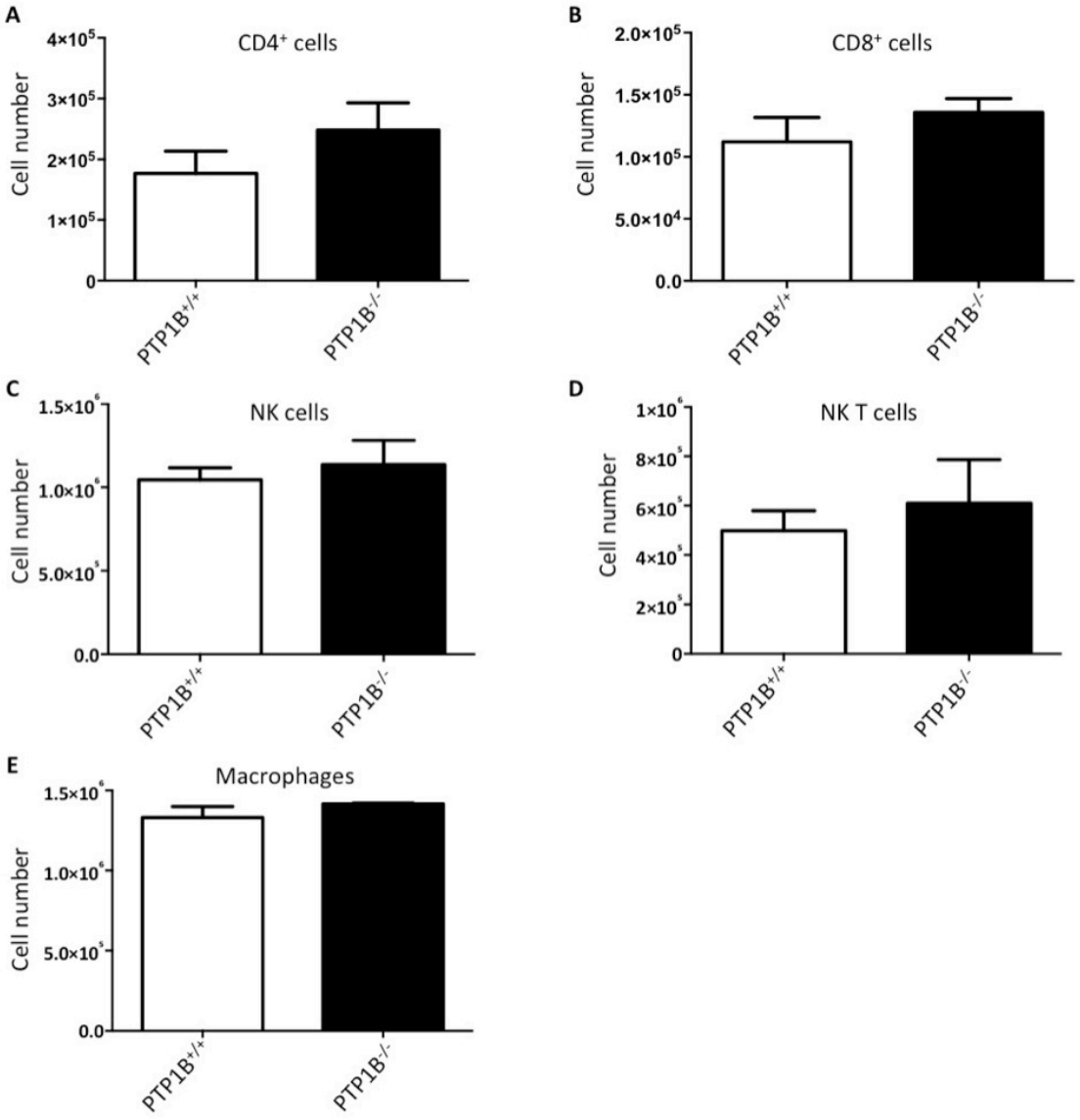


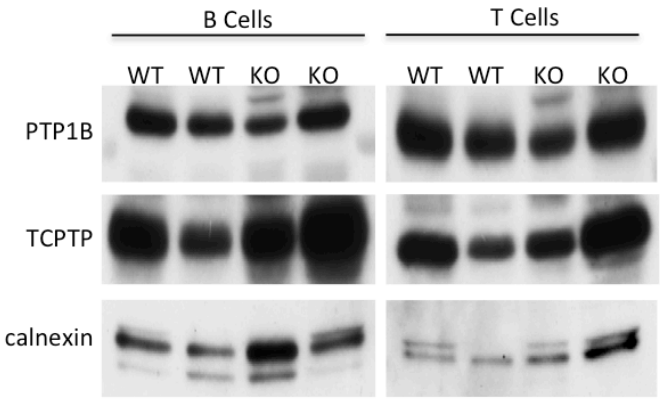
Figure 3.7 Dendritic cell specific deletion of PTP1B results in decreased survival in mice challenged with Eμ-myc lymphomas. A) Western blot analysis of BMDCs derived from *Ptpn1*$^{fl/fl}$-CD11cCre and *Ptpn1*$^{wt/wt}$-CD11cCre probed for PTP1B and the closely related TCPTP shows the efficiency and specificity of deletion. **B**) Kaplan-Meier analysis of survival of tumor bearing mice harboring CD11cCre DC tissue specific deletions of *Ptpn1* gene ***p<0.0001, *Ptpn1*$^{fl/fl}$-CD11cCre (n=9) and *Ptpn1*$^{wt/wt}$-CD11cCre (n=9). **C-D**) Lymphoma progression was monitored by *in vivo* bioluminescence imaging taken at days (7, 11, 13, 15 & 18) until saturation of the image or mouse endpoint precluded further monitoring. Representative images of differences in tumor dissemination between three *Ptpn1*$^{fl/fl}$-CD11cCre and *Ptpn1*$^{wt/wt}$-CD11cCre mice on Day 15 are shown.



Supplemental Figure 3.8 Survival analysis of mice injected with Eμ-myc lymphoma cells. A-D) Male six to ten week old WT or PTP1B$^{-/-}$ C57bl/6n mice were challenged via tail vein injections with either 10^6, 10^5, 10^4 or 10^3 syngeneic Eμ-myc lymphoma cells as depicted. Mice were monitored for tumor formation and progression via palpation of the inguinal and axillary lymph nodes. Kaplan-Meier analysis of survival of tumor bearing mice (n=3-12) harboring WT or deletion of *ptpn1* gene is shown.



Supplemental Figure 3.9 Analysis of immune cells infiltrating Eμ-myc lymphomas. WT and PTP1B null mice received subcutaneous injections of 10^6 Eμ-myc B cell lymphomas mixed in Matrigel. Implants were then surgically removed after 7 days, dissolved to single cell suspensions, and analyzed by flow cytometry for the presence of **A-B**) $CD4^+$ or $CD8^+$ T cells, **C-D**) NK or NKT cells, or **E**) macrophages.



Supplemental Figure 3.10 Tissue specificity of *Ptpn1*-CD11cCre mice. Spleens were harvested from two *Ptpn1*$^{fl/fl}$-CD11cCre and two *Ptpn1*$^{wt/wt}$-CD11cCre mice, B and T cells were isolated by either CD45 or CD3 negative selection respectively. Cells were then lysed and analyzed by western blot probing for PTP1B, the closely related phosphatase TC-PTP and calnexin as loading control.

3.5 Experimental Procedures

Tumor cell preparation

Tumors were aseptically removed and pooled from the lymph nodes of Eμ-myc transgenic mice [347]. The tumors were homogenized between two frosted glass slides, filtered and washed twice with sterile PBS before being resuspended to $1x10^6$ cells/ml. For *in vitro* culturing the cells were placed on a monolayer of either gamma-irradiated or mitomycin C (Sigma) treated mouse embryonic fibroblasts and maintained as previously described [348, 349].

Generation of a stable luciferase tumor cell line

Eμ-myc lymphoma cells were placed in culture and subsequently infected, as described [350], with a murine stem cell virus (MSCV) based IRES-GFP vector (Addgene #19360), subcloned with a firefly luciferase gene (Addgene #18751) into the multi-cloning site using BglII and XhoI restriction sites. Primer sequences were as follows: Luc-BglII GAGAAGATCTACCATGGAAGACGCCAAAAACATAA and Luc-XhoI GAGACTCGAGTTACACGGCGATCTTTCCGCC. GFP positive lymphomas were isolating using fluorescence activated cell sorting (FACS) (BD FACSaria sorter). Pure populations of GFP-luciferase expressing cells were expanded after sorting and frozen until needed.

Mouse studies

All animal procedures were carried out using 6-10 week old male C57Bl/6n mice according to the Canadian Council on Animal Care ethical regulations and were approved by the McGill University Research and Ethics Animal committee. $PTP1B^{-/-}$ mice were generated as previously described [148]. For the injection studies a 100 μl PBS suspension consisting of 100 GFP-luciferase expressing lymphoma cells plus 5×10^5 syngeneic splenocytes were injected into the tail veins of either $PTP1B^{-/-}$ or WT 6-10 week old males as described [350]. Mice were then monitored for lymphoma development by palpation of the inguinal and axillary lymph nodes and via whole body

luminescence imaging using the IVIS 100 in vivo imaging system (PerkinElmer). In vivo tumor cell proliferation was determined by calculating the total flux (photons/second) per animal over the duration of the study. *Ptpn1*$^{fl/fl}$ mice were obtained from the laboratory of Dr. Gerard Karsenty [351]. Dendritic cell specific deletion of *Ptpn1* was obtained by crossing *Ptpn1*$^{fl/fl}$ mice with Cd11c-Cre transgenic mice (The Jackson Laboratory).

Mouse cytokine profiles

Mouse cytokine and chemokine profiles were performed on mouse serum obtained by performing CO_2 euthanasia followed immediately by cardiac puncture. Blood was then centrifuged in blood collection tubes (Sarstedt) to obtain serum, which was stored at -20 deg. C until use. 500ul of mouse serum was used to interrogate the levels of 40 different cytokines and chemokines in a mouse cytokine proteome profiler antibody array (R&D systems) according to the manufacturers protocol. Radiographs were scanned on a Gel Doc system (Biorad) and quantified according to intensity. Results were analyzed and statistics performed using Graphpad Prism (GraphPad Software Inc.)

Immune infiltration assay

For immune infiltration analysis, 1x10^6 GFP-luciferase infected Eμ-myc lymphoma cells were mixed with 400 µl Matrigel (BD Biosciences) at 4°C and injected subcutaneously in either PTP1B$^{-/-}$ or PTP1B$^{+/+}$ mice. Implants were surgically removed after 7 days and incubated for 90 minutes with a solution of 1.6 mg/mL collagenase type IV (Sigma-Aldrich) and 200 µg/mL DNaseI (Sigma-Aldrich) in PBS as reported previously [329]. After incubation with anti-FcR III/II mAb (Biolegend) for 1 h, infiltrated cells were incubated for 1 hour at 4°C with antibodies as indicated: CD4, CD8, DCs: CD11c, IA/IE, H-2D^b, MDSC: CD11b, GR-1, Treg: CD4, CD25, FoxP3 (Biolegend) and analyzed by flow cytometry using a FACS Calibur cytometer (BD) [328].

Generation of murine PTP1B deficient BMDCs

Murine monocytes were isolated from the bone marrow of PTP1B$^{-/-}$ or corresponding WT littermates (WT moDCs) by CD11b positive selection using the EasySep® mouse monocyte CD11b positive selection kit (StemCell technologies). Monocytes were cultured with GM-CSF and IL-4 (20 ng/ml) (Peprotech) for six days to allow their differentiation into immature moDCs. On day six, immature moDCs were stimulated with 1ug/ml LPS (Sigma) for an additional 24 hours. On day seven supernatants and cells were collected.

Phenotypic analysis of BMDCs

Mature DC cell culture supernatants were tested for the levels of IL-6, IL-10 and IL-12 using enzyme-linked immunosorbent assay (ELISA) kits (Biolegend). Mature DCs were either collected and stored at -80°C for western blot analysis, or stained immediately for flow cytometry. The differentiation and maturation status of DCs was determined via labeling with fluorescence-conjugated antibodies specific for MHC class I and class II, CD40, CD80, CD86 and CD205 (Biolegend). The expression of these cell surface markers was determined by flow cytometry and analyzed using FlowJo software (TreeStar). To assay T cell activation, mature and OVA-pulsed DCs derived from WT, Het or KO PTP1B mice were co-cultured with either splenic purified CD8^{+} or CD4^{+} T cells isolated from OT-1 or OT-II transgenic mice respectively (Charles River). The cells were co-cultured for 48 hours and the amount of secreted IL-2 was determined in the supernatant.

For western blot analyses, protein samples were resolved on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblotting. Phospho-specific and total JAK2, STAT1, STAT3, STAT4 and STAT5 proteins were detected using polyclonal antibodies (Cell Signalling Technology). β-Actin (Sigma) was used as a loading control and PTP1B (BD) was detected with a monoclonal antibody (BD). Primary antibodies were followed by horseradish peroxidase-conjugated

goat anti–rabbit or mouse IgG (Jackson ImmunoResearch Laboratories). Blots were revealed using Western lightning chemiluminescent substrate (Perkin Elmer).

Statistical analysis.

Statistical analysis was performed by Prism 6 (Graph- Pad Software). The applied tests are indicated in the figure legends.

3.6 Discussion

The balance between immune suppression and immune surveillance is governed by the maturation state and activity of DCs as well as several other immune cell types such as $CD4^+$ and $CD8^+$ T lymphocytes, Tregs and macrophages [352-354]. Mature DCs are ideally equipped to present tumor antigens to effector T cells and elicit a host of anti-tumor immune responses. On the other hand when present in an immature state DCs can act to induce a potent tolerogenic and immune suppressive effect. Herein, we detail how PTP1B is required for proper maturation of DCs. PTP1B knockout mice when challenged with a syngeneic Eμ-myc model of B cell lymphoma are not able to mount a proper immune response leading to increased tumor cell proliferation and decreased survival. The DCs derived from these mice presented a dramatic phenotypic change characteristic of immature and tolerogenic DCs leading to immune suppression at the expense of anti-tumor immunity. Interestingly a recent study by Medgyesi et al. [355] has demonstrated that PTP1B negatively regulates CD40, B cell activating factor receptor and TLR4 signalling in B cells resulting in systemic autoimmunity. These findings highlight a growing trend necessitating proper PTP function as pivotal to the generation of a functional immune system. These dramatic changes we have described were in the context of heightened dysregulation of the key immunomodulatory JAK-STAT signalling cascades. PTP1B has been shown to target key components of this signalling axis and we specifically show increased activation of JAK2, STAT3 and STAT5 upon PTP1B deletion.

By utilizing the Eμ-myc mouse model of B cell lymphoma we were able to analyze the effect of PTP1B on the immune response to tumor challenge. PTP1B deficiency in mice results in decreased survival rates (**Figure 3.1**) revealing an important role for PTP1B in controlling lymphoma development. Similar tumor suppressive roles for PTP1B have previously been established in esophageal cancer and lymphoma [266, 356]. The dual nature of PTP1B in cancer has been largely attributed to the diversity of the various substrates PTP1B can target [262, 312]. Interestingly most of the published reports have detailed a cell autonomous role for PTP1B but have not considered the systemic effect PTP1B loss plays on the immune systems interaction with the tumor.

Since we noted a dramatic difference in the survival rates of mice deficient in PTP1B upon lymphoma challenge we were interested in potential alterations that may be present in the immune response to tumor challenge. Immune components of the PTP1B deficient mice, pro-inflammatory cytokines and chemokines, were dramatically altered compared to WT counterparts (**Figure 3.2**). PTP1B null mice as previously stated have been characterized as having systemic inflammation owing to preferential activation and expansion of macrophages. Our analysis further supports a role for PTP1B in regulating the inflammatory state [297, 318]. Despite the dramatic increases in serum cytokine levels PTP1B null mice do not develop spontaneous tumors, or other overt pathologies. Therefore, it suggests that it is only within the context of an external challenge such as the Eμ-myc lymphoma model, where one can really address if the immune system in PTP1B$^{-/-}$ mice becomes dysregulated.

Analysis of tumor infiltrates revealed increased numbers of immature DCs as well as increases in the number of the suppressive MDSCs and Tregs. In partial agreement with our observations, a recent study by Zhang et al. [334] has shown PTP1B deletion to enhance the MDSC population through regulation of the JAK2-STAT3 signalling pathway in an experimental model of colitis. To our knowledge, this study is the first to report a role for PTP1B in regulating DCs in a tumor model, although several reports exist

exploring the role of other phosphatases in DC function. Deletion of the PTP Shp-2 has been shown to regulate the auto-immunity function in DCs and promote Th1 polarization [357]. Considering that PTP1B and Shp-2 do not share the same substrates it is understandable that we did not observe the same effect. More recently the phosphatase PTP-PEST was shown to play an important role in DC migration and the induction of T cell responses [358]. PTP-PEST plays an important role in cell migration and cell-cell interaction and therefore its role in priming the immune response is likely due to an impairment in DC migration to the lymph nodes.

To better understand the effect PTP1B has on DCs we examined the phenotype of BMDCs derived from $PTP1B^{+/+}$ and $PTP1B^{-/-}$ mice *in vitro*. We noted significant differences in the expression of surface markers and costimulatory markers required for proper antigen presentation and activation of T cells (**Figure 3.4**). Proper expression of these molecules is characteristic of mature DCs and required for their ability to present antigen to T cells and elicit an anti-tumor response. The immunostimulatory properties of dendritic cells are directly linked to their maturation state. Injections of mature DCs rapidly enhances antigen-specific $CD4^+$ and $CD8^+$ immunity, however injections of immature DCs has reported to induce an immunosuppressive response [337]. This suppressive role has been correlated with increased recruitment and activity of regulatory T cells [59, 359]. It is likely that the immature DCs we have observed in our study are contributing to enhanced tumorigenesis via recruitment or activation of additional Tregs, however this is beyond the scope of the current study but warrants further investigation.

Signalling by the JAK2-STAT3 pathway has been shown to be critically important for DC differentiation [84]. Hyperactivation of STAT3 as we have observed has been directly shown to contribute to abnormal DC differentiation and accumulation of immature myeloid cells preventing the generation of mature DCs [360]. Aberrant STAT3 regulation is characteristic of many types of malignancies [361, 362] and removal of PTP1B from

DCs appears to mimic some of these effects. It is then possible to postulate that PTP1B could contribute to STAT3 tumorigenesis by virtue of the regulatory role it has been ascribed. However it is critical to recognize that there are cellular redundancy systems in place, which could compensate for an alteration in PTP1B activity. For example the closely related phosphatase TC-PTP has been also implicated in STAT3 regulation and plays an important role in breast cancer and macrophage development [289, 363] The STAT3 transcription factor has shown to be critical in the suppression of MHC II surface expression and activation of the $CD4^+$ T cell response [90, 327]. This signalling cascade is initiated through increased IL-6-STAT3 signalling leading to increased cathepsin activity resulting in reduced intracellular MHC II αβ dimmer, Ii, and H2-DM levels in DCs. Our analysis revealed similar enhancement of STAT3 activation and decreased MHC II surface expression and impairment in $CD4^+$ T cell activation. We did not detect any difference in IL-6 levels, however our observations support a model of direct JAK2-STAT3 hyperactivation directly downstream of receptor signalling.

In summary we have identified a novel role for PTP1B in the regulation of dendritic cell maturation and signalling. Mice lacking PTP1B both in the whole body and in the dendritic cell compartment exhibited decreased survival when challenged with an Eμ-myc model of B cell lymphoma. This survival defect was attributed to increased numbers of suppressive and immature dendritic cells infiltrating the tumor microenvironment. Dysregulation in dendritic cell biology was due to perturbations in the JAK2-STAT3 signalling pathway.

3.7 Acknowledgements

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CHAPTER 4: TARGETING PROTEIN TYROSINE PHOSPHASTASES AS THE BASIS FOR A NOVEL DENDRITIC CELL BASED IMMUNOTHERAPY

Claudia Penafuerte*, Matthew Feldhammer*, George Zogopoulos and Michel L. Tremblay (manuscript in preparation, *authors contributed equally to this work)

4.1 preface to the manuscript

PTP1B is a ubiquitously expressed protein tyrosine phosphatase that has recently been implicated in regulating immune signalling. The genetic deletion of PTP1B resulted in the accumulation of immature and tolerogenic DCs in an Eμ-myc mouse model of B cell lymphoma. DCs were impaired in their ability to activate T cell responses resulting in increased lymphoma progression and decreased survival (**chapter 2**). We were therefore interested in determining if by modulating the activity of PTP1B through either genetic manipulation or pharmacological inhibition we could enhance the expression of key DC maturation markers leading to more potent T cell responses.

4.2 abstract

The protein tyrosine phosphatases (PTP) 1B, and TC-PTP have been established as key regulators in macrophage activation and the inflammatory response. We have recently demonstrated that PTP1B deficient dendritic cells (DCs) were present in an immature tolerogenic state in an Eμ-myc lymphoma tumor model, leading to enhanced tumor proliferation and decreased overall survival. Here we show that by modulating the level of PTP1B and TCPTP we can increase DC maturation and T-cell activation. By using DCs derived from mice heterozygous for PTP1B and TC-PTP, as well as DCs treated with a PTP1B/TCPTP inhibitor, we measured decreased tumor progression in mice challenged with EG7-lymphoma. DCs heterozygous for PTP1B exhibit augmented expression of MHC class I on the cell surface and upregulated costimulatory molecule expression. Heterozygous PTP1B DCs produce higher levels of the key Th1 cytokine IL-12 and show an enhanced ability to activate $CD8^+$ T cells. Based on these findings we propose a DC based vaccine formulation to boost the capacity of cancer patients' derived DCs to induce the maturation and activation of T cells. Using a PTP1B/TCPTP inhibitor to treat DCs derived from metastatic pancreatic cancer patients we were able to increase IL-12 production as well as antigen-specific $CD8^+$T cell activation. Our results provide the rationale for modulation of PTP1B/TC-PTP activity as a means of increasing DC maturation and eliciting tumor clearance in a novel DC based vaccine formulation.

4.3 Introduction

Dendritic cell (DC) based vaccines are the subject of active investigation for the treatment of many human malignancies. Owing to their capacity to activate potent tumor antigen-specific immunity, DCs are prime candidates for the development of anti-cancer vaccines. In contrast to monoclonal antibody and cytokine-based therapies, DC vaccines are relatively well tolerated by patients and produce few side effects [364, 365]. In addition to priming potent Th1 directed $CD4^+$ T cell responses, DCs can cross-present exogenously derived peptide antigens to activate naive $CD8^+$ cytotoxic T lymphocytes (CTLs) [26]. Despite the potential to induce potent tumor antigen-specific T cell responses, efforts to develop DC based vaccines for cancer have only resulted in a single therapy receiving regulatory approval. A major challenge in the generation of DC based anti-tumor immunity is the lack of key activation signals in the tumor microenvironment preventing proper maturation of DCs and promoting immune tolerance [6]. Increased numbers of immature and tolerogenic DCs have been observed in cancer patients and can lead to immune suppression and the promotion of tumor growth [46, 336, 366].

Three major DC-derived signals dictate the fate of T cell responses. The first signal provides antigen specificity by the recognition of antigenic peptides presented by major histocompatibility complexes (MHC class I/II). The second signal, driven by the costimulatory molecules is crucial in determining T cell response; and the third signal is mediated by DC-derived cytokines, which imprints a polarizing T cell program towards either immunity or tolerance [59, 367]. The maturation state of DCs is therefore crucial in the development of T cells into either tolerogenic or cytotoxic effector cells. Mature DCs upregulate the expression of MHC class I and class II, costimulatory molecules and cytokines such as IL-12, this being the most distinguished feature of fully mature and immunogenic DCs [332]. IL-12 is important for resistance to cancer development and progression. DCs over-expressing IL-12 for cancer therapy showed greater NK cell activation and effector function as well as an increase of tumor associated antigen

(TAA)-specific CTLs [368]. IL-12 can induce the expression of the cytolytic enzymes perforin and granzyme, promoting the development of CTLs and lymphocyte-activated killer cells that have the ability to destroy tumor cells.

Several studies indicate that the impaired DC function in cancer patients is linked in part to dysregulation of the janus activated kinase (JAK) family of tyrosine kinases and signal transducer and activator of transcription (STAT) proteins [346, 369]. Many tumor-derived factors take advantage of this pathway to promote abnormal DC differentiation by inducing STAT3 hyperactivation [360]. The two highly related protein tyrosine phosphatases (PTPs) TC-PTP and PTP1B, are major negative regulators of JAK/STAT signalling pathways, suggesting that these enzymes may also regulate DC maturation and activation [370]. PTP1B and TC-PTP are the two of the most well defined members of the class I subfamily of tyrosine specific PTPs. Both PTPs are ubiquitously expressed and share ~75% sequence homology in their catalytic domains resulting in overlap in some of their substrate recognition profiles [312, 370, 371]. Recently, we have shown that DC maturation was compromised upon deletion of PTP1B (**Chapter 3**). Genetic deletion resulted in hyperactivation of JAK2, STAT3 and STAT5 signalling resulting in a decrease of MHC class II and costimulatory CD40 expression. Dendritic cells derived in the absence of PTP1B secreted less IL-12 and were defective in their ability to activate $CD4^+$ T cells. These results were the first to establish a role for PTP1B in the regulation of DC maturation.

Based on our findings we were interested in determining if, by modulating PTP1B activity, we could enhance the expression of key DC maturation markers resulting in increased anti-tumor immunity. We report herein that modulating PTP1B and TC-PTP activity through either genetic or pharmacological means results in the preferential maturation of DCs capable of eliciting potent anti-tumor immunity. These results in animal models as well as human pancreatic cancer patient derived DCs provide the

rationale for the development of a novel DC based cancer immunotherapy targeting both PTP1B and TC-PTP.

4.4 Results

4.4.1 PTP1B heterozygous DCs display increased expression of key maturation markers.

Our lab has previously shown that a PTP1B inhibitor is able to confer long lasting protection in an ErbB2 model of breast cancer even weeks after the inhibitor is removed [267]. This suggests that by modulating PTP1B activity you could provide long lasting tumor protection, possibly due to an enhancement of anti-tumor immunity. In order to determine if the modulation of PTP1B activity could lead to enhanced DC maturation we first investigated the phenotype of bone marrow derived DCs (BMDCs) from PTP1B heterozygous mice. In agreement with our previous results, we saw a significant reduction in MHC class II expression in the $PTP1B^{-/-}$ mice (**Figure 4.1 A; Chapter 3 Figure 3.4 B**). We observed no differences in the expression levels of MHC class II between the $PTP1B^{+/-}$ and the $PTP1B^{+/+}$ mice (**Figure 4.1 A**). $CD4^+$ T cells recognize antigens coupled to MHC class II on DCs, whereas antigenic peptides are presented in the context of MHC Class I to $CD8^+$ T cells. We observed significant upregulation of MHC class I expression (**Figure 4.1 B**) between $PTP1B^{+/-}$ and $PTP1B^{+/+}$ BMDCs, as well increased expression of the costimulatory molecule CD86 in the $PTP1B^{+/-}$ BMDCs (**Figure 4.1 C**). Immature DCs are characterized by low expression of MHC and costimulatory molecules. As DCs undergo maturation, expression of MHC class I, class II as well as costimulatory molecules are upregulated in addition to secretion of high levels of the key Th1 promoting cytokine IL-12. Consequently, mature DCs are highly capable of inducing antigen-specific T cell immunity and effective tumor clearance. Our results here confirm that by modulating the expression of PTP1B we were able to enhance the expression of key surface molecules required for $CD8^+$ T cell activation.

IL-12 production is the third stimulatory signal essential for T cell activation and development of CTLs. We used an enzyme linked immunosorbent assay (ELISA) to

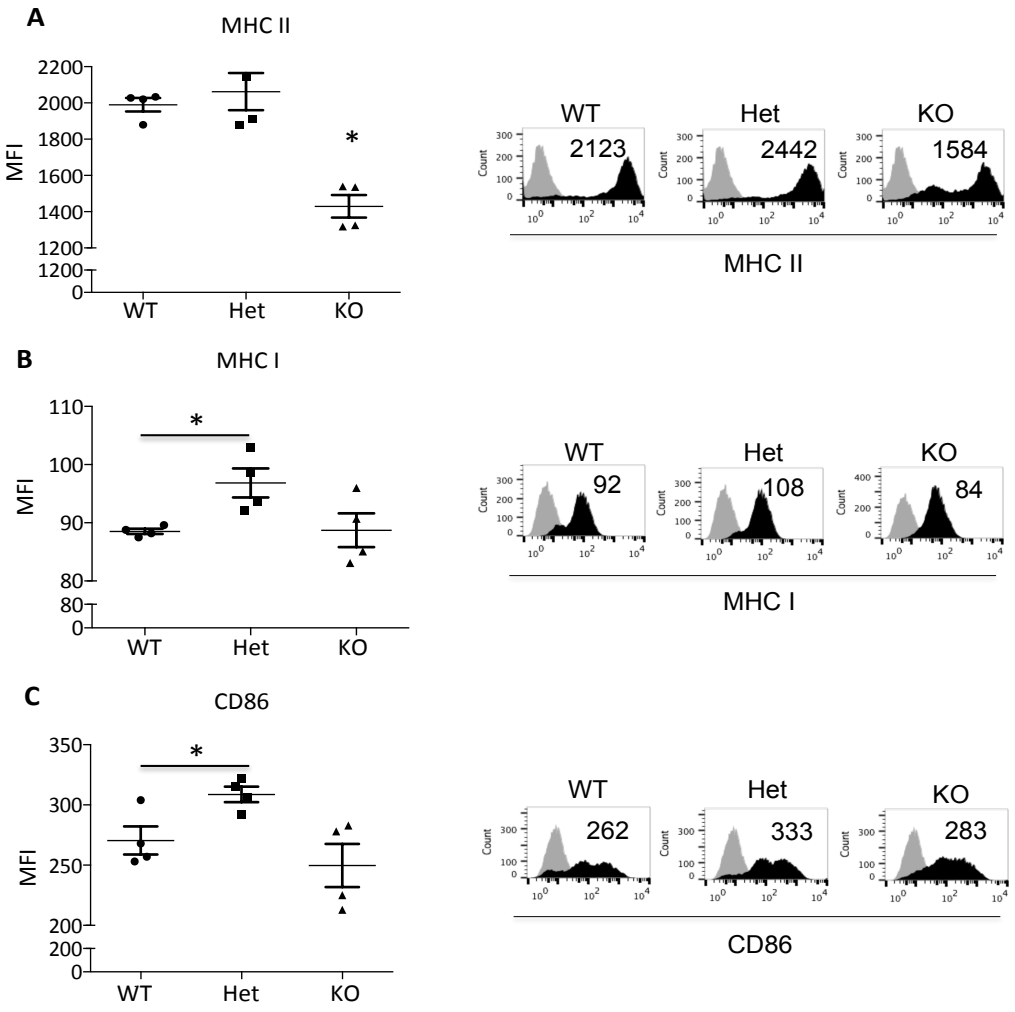

Figure 4.1: PTP1B heterozygous DCs display increased expression of key maturation markers. A-E) Expression of maturation markers on mature PTP1B KO, Het and WT DCs. Mouse bone marrow monocytes were surgically extracted from femurs and tibias. Monocyte enrichment using CD11b positive selection was performed and $15x10^6$ monocytes were cultured for 7 days before maturation was induced with LPS to obtain mature BMDCs. Cells were then stained for CD11c and the indicated markers with specific fluorescence conjugated antibodies and their expression upon maturation was determined by flow cytometry. Mean fluorescence intensity (MFI) of MHC class I, MHC class II, CD86 expression and histograms of one representative sample for each marker respectively (n=4). Significant differences are represented by asterisk as determined by students T test (*P* values <0.05). Results are representative of two independent experiments.

quantify the amounts IL-12 secreted by BMDCs derived from PTP1B$^{+/-}$ mice. We also measured the levels of the potent immune suppressive cytokine IL-10. We observed significant increases in the amount of IL-12 secreted by PTP1B$^{+/-}$ BMDCs (**Figure 4.2 A**). In agreement with our previous study, we also noted decreased IL-12 secretion in the PTP1B$^{-/-}$ BMDCs (**Figure 4.2 A**; **Chapter 3, Figure 3.5 A**). We noted no differences in the amount of the inhibitory cytokine IL-10 between PTP1B$^{+/-}$ and PTP1B$^{+/+}$ BMDCs. These results demonstrate that DCs heterozygous for PTP1B have increased expression of the key markers of DC maturation, MHC Class I, CD86 and the Th1 polarizing cytokine, IL-12.

Based on the more immunogenic phenotype observed in PTP1B heterozygous BMDCs, we hypothesized that PTP1B$^{+/-}$ BMDCs would be more efficient at antigen presentation to CD8^{+} T cells resulting in their increased activation. Co-culture assays were carried out in order to assess the CD4^{+} and CD8^{+} activation potential of PTP1B$^{+/-}$ BMDCs. Mature and chicken ovalbumin (OVA)-pulsed BMDCs derived from PTP1B$^{+/+}$, PTP1B$^{+/-}$ and PTP1B$^{-/-}$ mice were co-cultured with OVA-specific naïve CD8^{+} and CD4^{+} T cells derived from OT-1 and OT-2 mice respectively. The level of secreted IL-2 was measured as a marker for T cell activation. We observed significant increases in CD8^{+} T cell activation in cells cultured with PTP1B$^{+/-}$ BMDCs (**Figure 4.2 C**) which correlates with their increased expression of MHC class I, CD86, and IL-12 secretion. We did not observe differences in the ability of PTP1B$^{+/-}$ BMDCs to activate CD4^{+} cells when compared with PTP1B$^{+/+}$ BMDCs (**Figure 4.2 D**). We did note a significant decrease in the ability of PTP1B$^{-/-}$ BMDCs to active CD4^{+} T cells compared with the PTP1B$^{+/+}$ BMDCs further confirming our earlier observations (**Figure 4.2 D**; **Chapter 3 figure 3.5 E**)

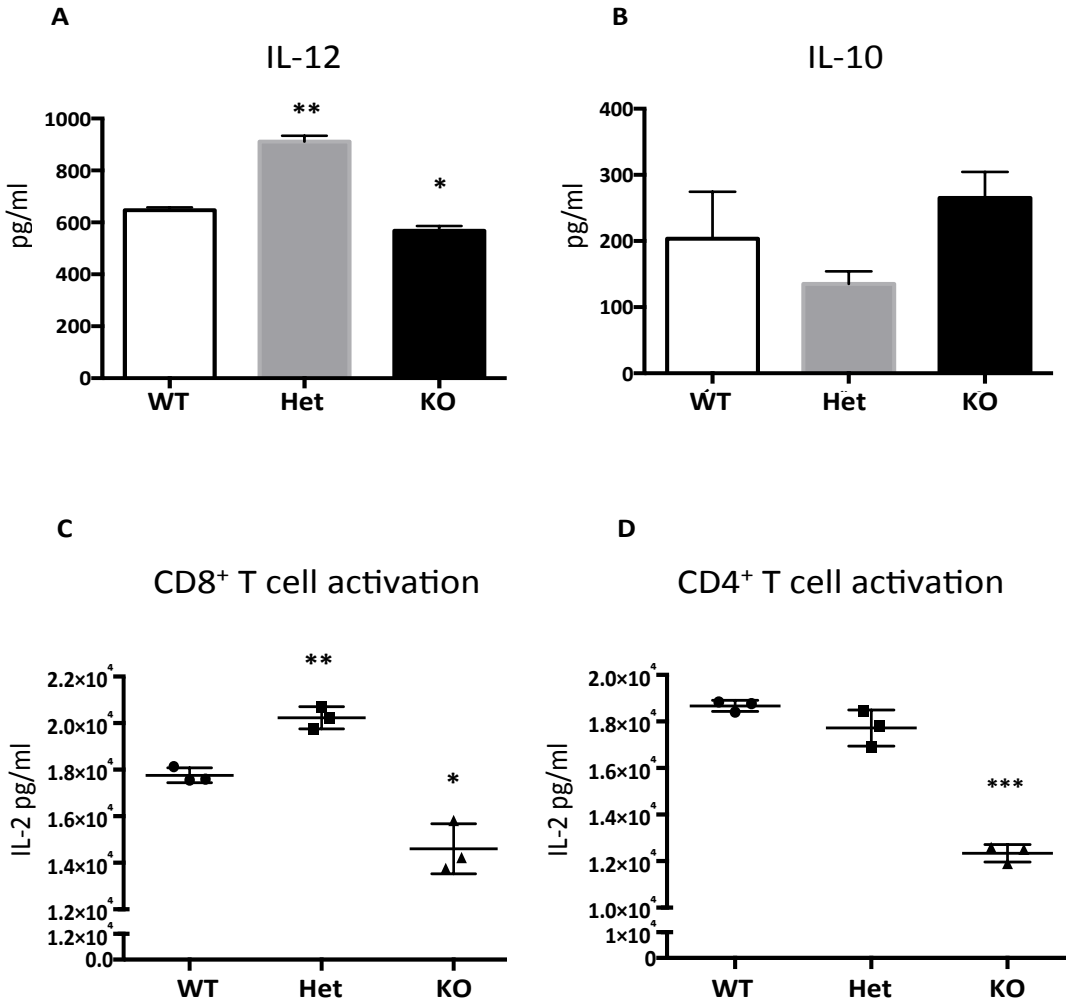

Figure 4.2: PTP1B heterozygous DCs secrete more IL-12 and display enhanced CD8$^+$ T cell activation. BMDCs were derived as in Figure 4.1 from PTP1B$^{+/+}$, PTP1B$^{+/-}$ and PTP1B$^{-/-}$ mice. Maturation was induced with LPS for 24 hours at which point, supernatants were collected and frozen until use. Supernatants were then tested for the presence of IL-12, and IL-10 cytokine production by ELISA as indicated. Data are shown as the mean of two independent experiments for IL-12 and one experiment for IL-10, each with n=3-6. **C-D)** LPS + OVA stimulated DCs from WT, Het or KO PTP1B BMDCs were co-cultured with either OT-1 expressing CD8+ or OT-II CD4+ T cells for 48 hours and the amount of secreted IL-2 was determined. Significant differences are represented by asterisk (n=3, results are representative of two independent experiments. Significant differences are denoted by asterisks as determined by Mann-Whitney tests, ***, $P < 0.0005$, **, $P<0.005$; *, $P<0.05$

4.4.2 *Mice treated with PTP1B$^{+/-}$ DCs display significantly delayed tumor growth*.

Based on our observations that BMDCs from PTP1B$^{+/-}$ mice displayed an increased expression of the key markers of DC maturation and had an enhanced ability to activate CD8^{+} T cells, we wanted to investigate their capacity to function as a cell-based anti-cancer vaccine. We subcutaneously implanted mice with syngeneic OVA-expressing EG7-OVA lymphoma cells and allowed the tumors to grow for ten days. On day ten it was determined that mice had similar tumor burden based on caliper measurements (**Figure 4.3 A**). Mice were then divided into four groups to receive either intraperitoneal injections of BMDCS from PTP1B$^{+/+}$, PTP1B$^{+/-}$ or PTP1B$^{-/-}$ syngeneic mice, or no BMDCs as a control. Tumor growth was monitored via caliper measurements (**Figure 4.3 A**) or by bioluminescence (**Figure 4.3 B-D**) until the control group tumor burden reached endpoint and the mice were sacrificed. PTP1B$^{+/-}$ BMDC treated mice had significantly delayed tumor growth throughout the duration of the study. This confirmed that by modulating the activity of PTP1B we were able to enhance DC based immunity and effectively slow tumor progression.

4.4.3 A PTP1B inhibitor enhances BMDC maturation by targeting both PTP1B and TC-PTP.

Based on the above observations made with PTP1B$^{+/-}$ BMDCs we hypothesized that PTP1B was ideally suited for pharmacological intervention in order to generate a novel DC-based cancer vaccine. We sought to determine if we could use a PTP1B inhibitor to induce a phenotype similar to what we had observed in the PTP1B$^{+/-}$ DCs. Inhibitor titration on BMDCs resulted in increases in IL-12 production, with a maximum observed at 5μM (**Figure 4.4 A**). We then generated BMDCs in either the presence or absence of the PTP1B inhibitor and observed significant increases in the expression of MHC class I, class II and the costimulatory molecules CD80 and CD86 (**supplemental Figure 4.7 A-D**). This indicated that the PTP1B inhibitor was able to reproduce the enhanced expression of key maturation markers we observed in PTP1B$^{+/-}$ BMDCs.

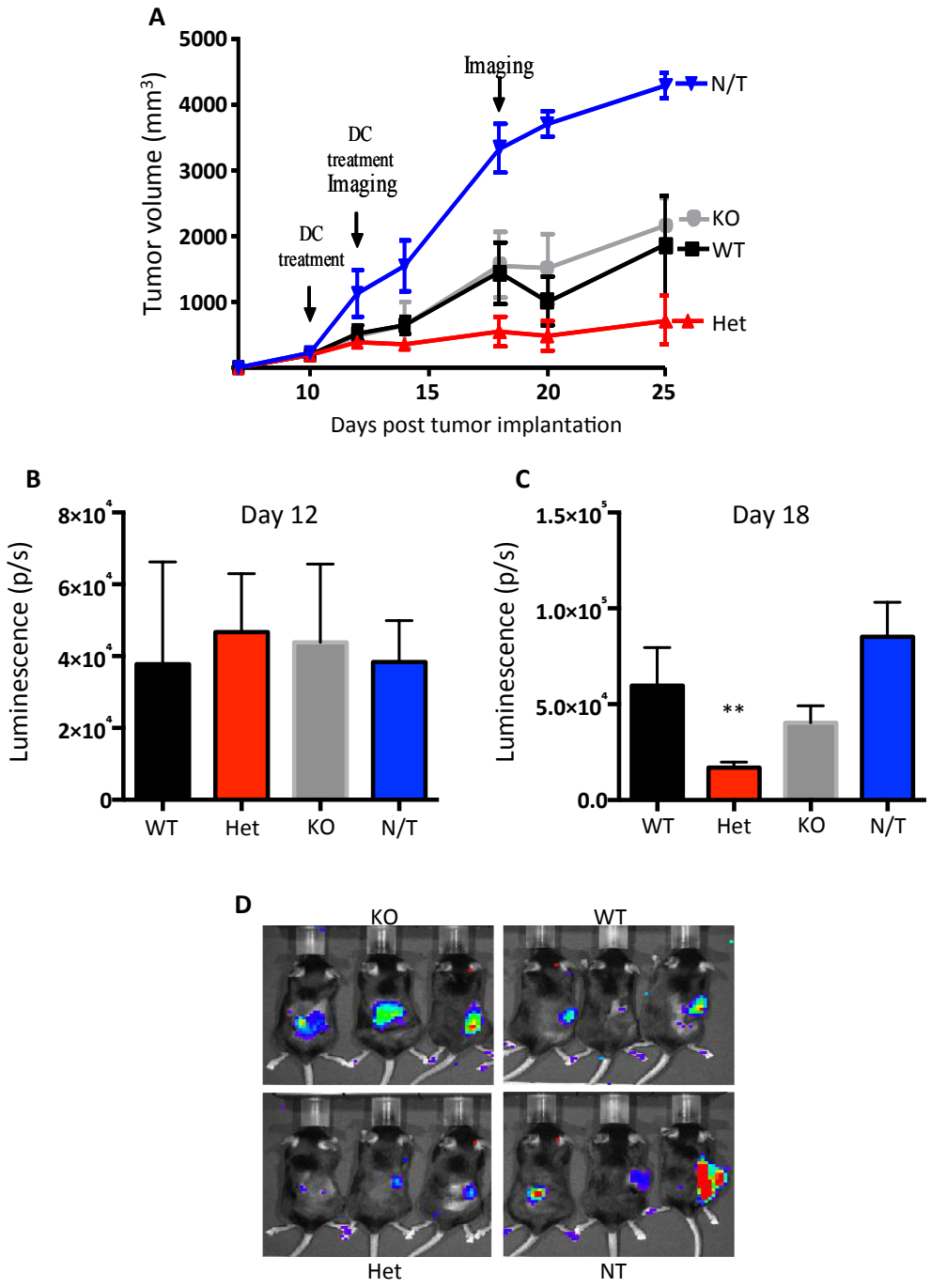

Figure 4.3: Mice treated with PTP1B$^{+/-}$ DCs display significantly delayed tumor growth.

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Figure 4.3: Mice treated with PTP1B$^{+/-}$ DCs display significantly delayed tumor growth. **(A)** Monitoring of Tumor growth over time of via caliper measurements of EG7-OVA lymphoma-bearing mice (subcutaneously injected with $5x10^5$ tumor cells) treated via intraperitoneal (IP) injection with $2x10^6$ OVA-pulsed and mature DCs derived from PTP1B$^{+/+}$, PTP1B$^{+/-}$ and PTP1B$^{-/-}$ mice (mice received DC treatments on day 10 and day 12). Measurement of tumor burden via *in vivo* bioluminescence at 12 (**B**) and 18 (**C**) days post tumor implantation. **D**) Representative images taken on day 18 post tumor implantation showing corresponding tumor burdens. Significant differences are represented by asterisk as determined by students T test (***P* <0.005, n=5). Results are representative of two independent experiments.

In order to assess inhibitor specificity, we utilized an *in vitro* fluorimetric assay with classical, receptor and non-receptor based PTPs (TC-PTP, SHP-1, Sigma and LAR) as well as the dual specificity phosphatase MKPX. It was determined that the inhibitor was not only specific for PTP1B, but for the closely related phosphatase, TC-PTP, as well (**Figure 4.4 B**). Therefore, it will be referred to as 'PTP inhibitor' throughout the rest of this thesis.

Because we observed an increased maturation phenotype in inhibitor treated BMDCs, we hypothesized that BMDCs from TC-PTP mice may behave in a similar fashion. To address this question we generated mice carrying a DC-specific deletion of TC-PTP. Using these *Ptpn2*-CD11cCre mice, we generated BMDCs and observed increased expression of MHC class I, class II as well as CD80 and CD86 in the heterozygous group (**Supplemental figure 4.7 E-H**). These results reveal that by modulating the activity of PTP1B and/or TC-PTP, either through genetic or pharmacological intervention, we can generate more mature and immunogenic DCs.

4.4.4 Therapeutic potential of PTP inhibitor treated BMDCs in a mouse lymphoma model.
PTP inhibitor treated BMDCs, as well as BMDCs from *Ptpn2*$^{wt/fl}$-CD11cCre mice, exhibited an enhanced maturation phenotype and should therefore be more effective in eliciting anti-tumor immunity. In order to assess the therapeutic potential of inhibitor treated BMDCs, mice were implanted subcutaneously with syngeneic EG7-OVA lymphoma cells and tumors were allowed to grow for ten days. As previously done, following ten days of tumor growth we determined that mice have similar tumor burdens as measured by bioluminescence (**Figure 4.5 A**). Mice were then divided into three groups and received either therapeutic injections of inhibitor-treated BMDCs, control non-treated BMDCs or no injections. Eleven days following therapeutic intervention, mice that received PTP inhibitor treated BMDCs exhibited significant delays in tumor growth (**Figure 4.5 B-C**). Because we determined that the PTP inhibitor also targeted TC-PTP we repeated the same experiment using BMDCs derived from *Ptpn2*-CD11cCre mice. Ten days following

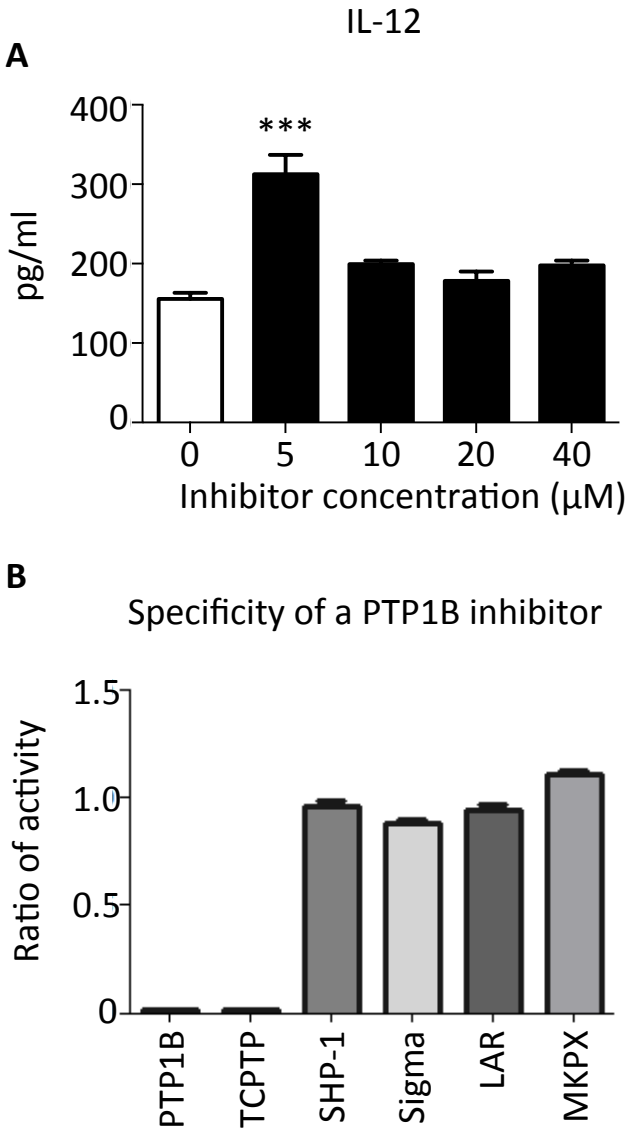


Figure 4.4: A PTP1B inhibitor enhances BMDC maturation by targeting by PTP1B and TC-PTP (A) Titration of increasing concentrations of the PTP1B inhibitor into WT mouse DCs. Twenty-four hours post maturation via LPS, supernatants were harvested and IL-12 production was quantified by ELISA (n=4, results are representative of two independent experiments). **B**) The specificity of the PTP1B inhibitor against various protein tyrosine phosphatases was determined using DiFMUP assay. Significant differences are represented by asterisk as determined by students T test (****P* value <0.0005).

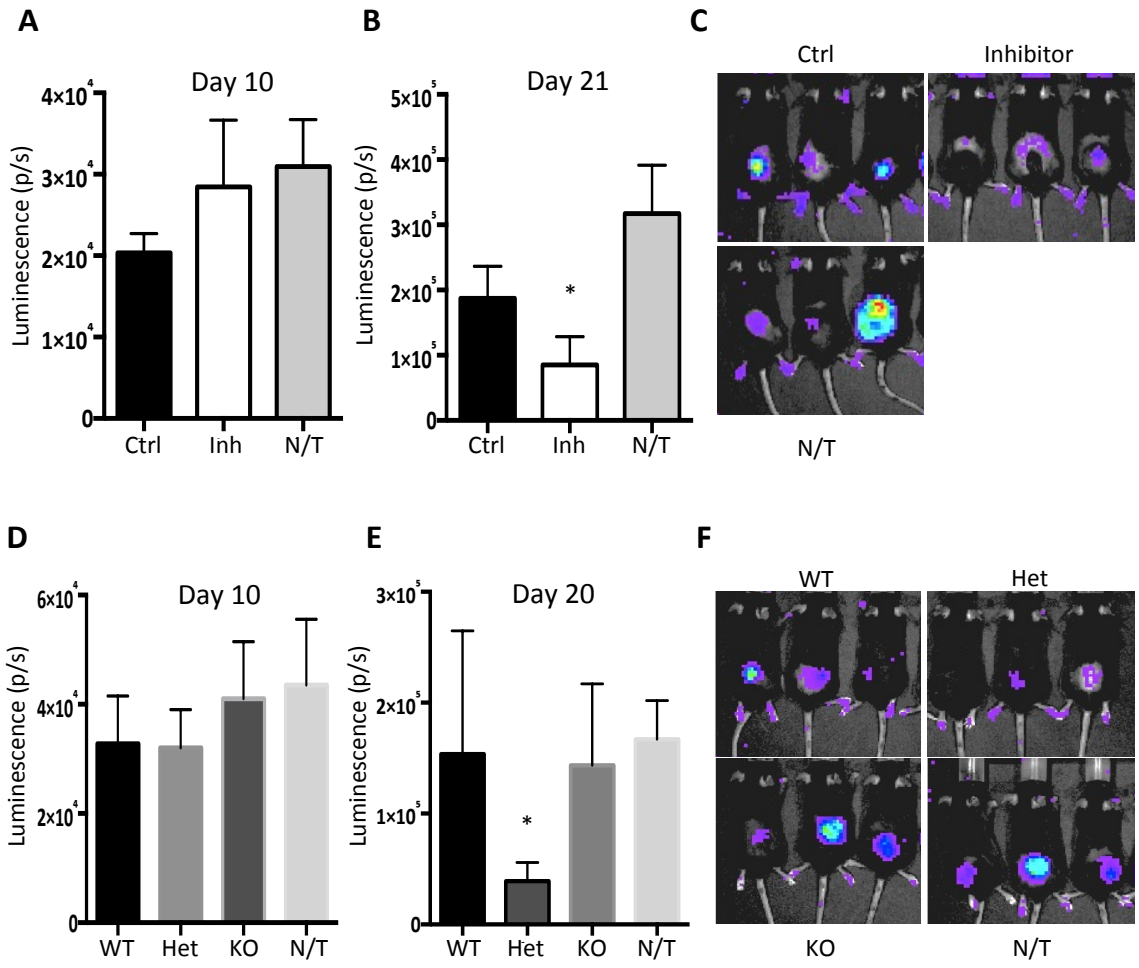


Figure 4.5: Therapeutic potential of PTP inhibitor treated BMDCs in a mouse lymphoma model. Figure legend on next page

Figure 4.5: Therapeutic potential of PTP inhibitor treated BMDCs in a mouse lymphoma model. EG.7-OVA lymphoma-bearing mice (subcutaneously injected with $5x10^5$ tumor cells) were treated via IP injection on day 10 with $5x10^6$ mature OVA-pulsed PTP inhibitor treated DCs (**A-C**) or WT, TC-PTP heterozygous or KO BMDCs (**D-F**) **A)** Measurements of tumor burdens at 10 days post tumor implantation and before injections with PTP inhibitor treated DCs. **B)** *In vivo* bioluminescence measurements of tumor burden at 21 days post tumor implantation and after injections with mature PTP inhibitor treated DCs. **C)** Representative images taken on day 21 post tumor implantation showing corresponding tumor burdens. Results are representative of two independent experiments **(D)** *In vivo* bioluminescence measurements of tumor burden at 10 days post tumor implantation and before treatment with WT, TC-PTP heterozygous or KO DCs. **(E)** Tumor burdens at 20 days post tumor implantation and after treatment with TCPTP DCs. **(F)** Representative images taken on day 20 post tumor implantation showing corresponding tumor burdens. Significant differences are represented by asterisk (*P* values <0.05) n=5.

therapeutic intervention, mice that received heterozygous TC-PTP BMDCs also exhibited a significant delay in tumor progression (**Figure 4.5 D-E**). To investigate the ability of heterozygous TC-PTP BMDCs to act as a prophylactic anti-cancer vaccine, we immunized *Ptpn2*$^{wt/wt}$-, *Ptpn2*$^{wt/fl}$- or *Ptpn2*$^{fl/fl}$- CD11cCre mice with OVA antigen prior to EG7-OVA lymphoma challenge. Mice that had heterozygous deletions of TC-PTP in their DCs were protected for over 100 days following tumor implantation **Supplemental Figure 4.8 A-C**). After a year, the tumor-free mice were sacrificed to terminate the experiment. These results confirmed that by modulating the activity of PTP1B and/or TC-PTP we can effectively elicit anti-tumor immunity and slow tumor growth.

4.4.5 Generation of a DC based immunotherapy for use in advanced human pancreatic cancer.

Pancreatic cancer (PaC) has one of the lowest five year survival rates of all cancer types [372]. Due a lack of effective early screening methods, the majority of PaC patients are at an advanced stage of disease at the time of diagnosis and are therefore ineligible for surgical interventions [373]. Standard treatment regimens involving radiation and chemotherapy are generally considered palliative and provide only modest survival increases [374]. DC-based immunotherapies therefore represent a promising new therapeutic approach and may provide some benefit to patients undergoing standard treatment protocols [375-377]. In order to evaluate the potential clinical benefit of a PTP inhibitor based DC immunotherapy, we generated monocyte-derived DCs from advanced Pac patient peripheral blood and evaluated the ability of PTP inhibitor treated DCs to produce IL-12 and activate tumor antigen-specific T cells. Patient DCs were primed with CEA and CA19-9 as tumor-associated antigens (TAAs) (**Figure 4.6 A**) as detailed in the methods section. Inhibitor treated PaC patient DCs displayed significantly increased capacities to produce IL-12 compared with non-treated DCs (**Figure 4.6 B**). We subsequently evaluated the ability of inhibitor treated PaC DCs to activate T cells in an ELISpot assay. Autologous patient T cells were incubated with either inhibitor treated or non-treated DCs for 48 hours and the amount of secreted IFN-γ was quantified. Two out

of the three patients tested exhibited significant increases in TAA-specific T cell activation when compared with non-inhibitor treated controls (**Figure 4.6 C**). These results provide the basis for further evaluation of PTP inhibitor treated DCs as a novel DC-based immunotherapy approach for the treatment of cancer.

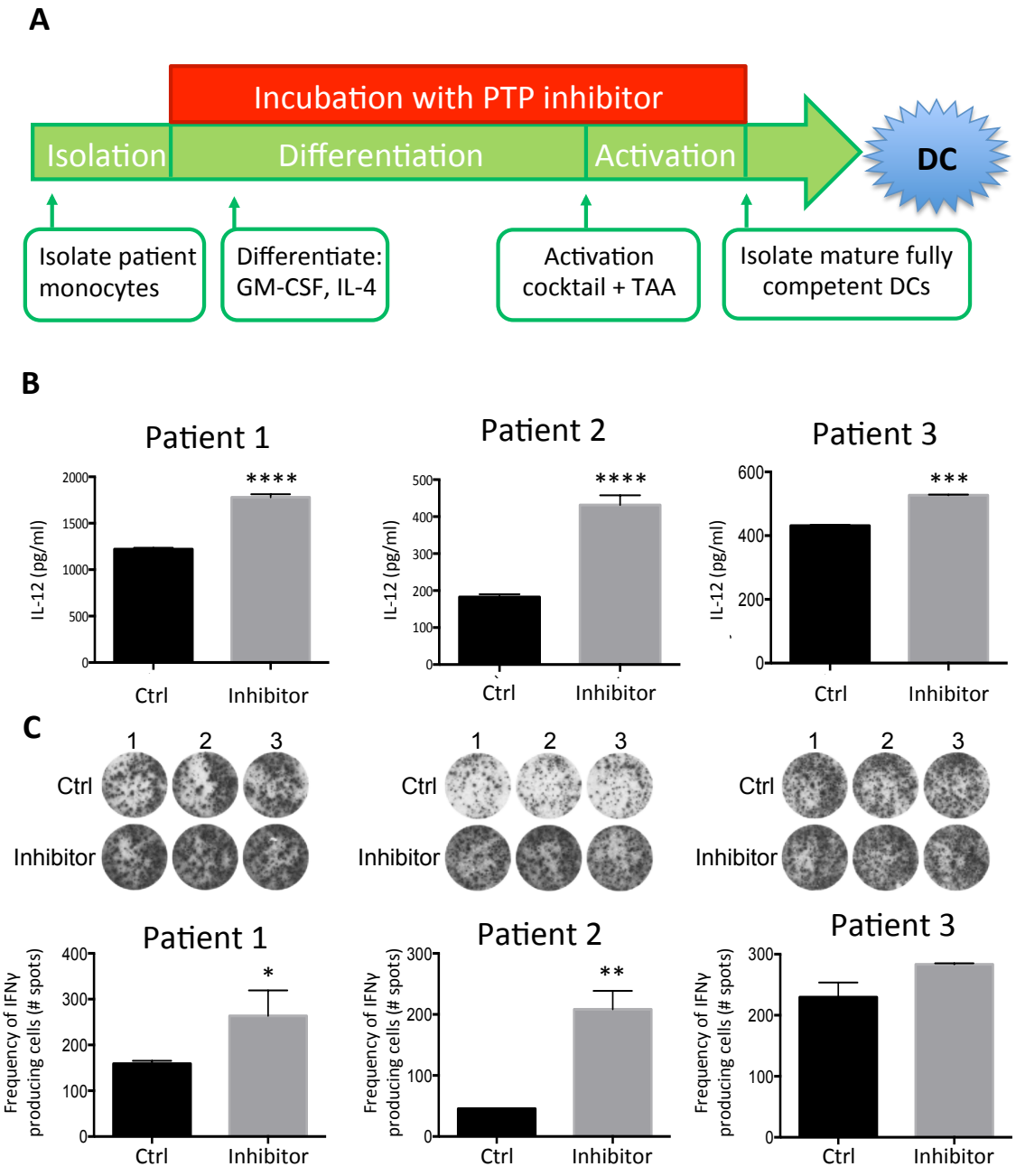
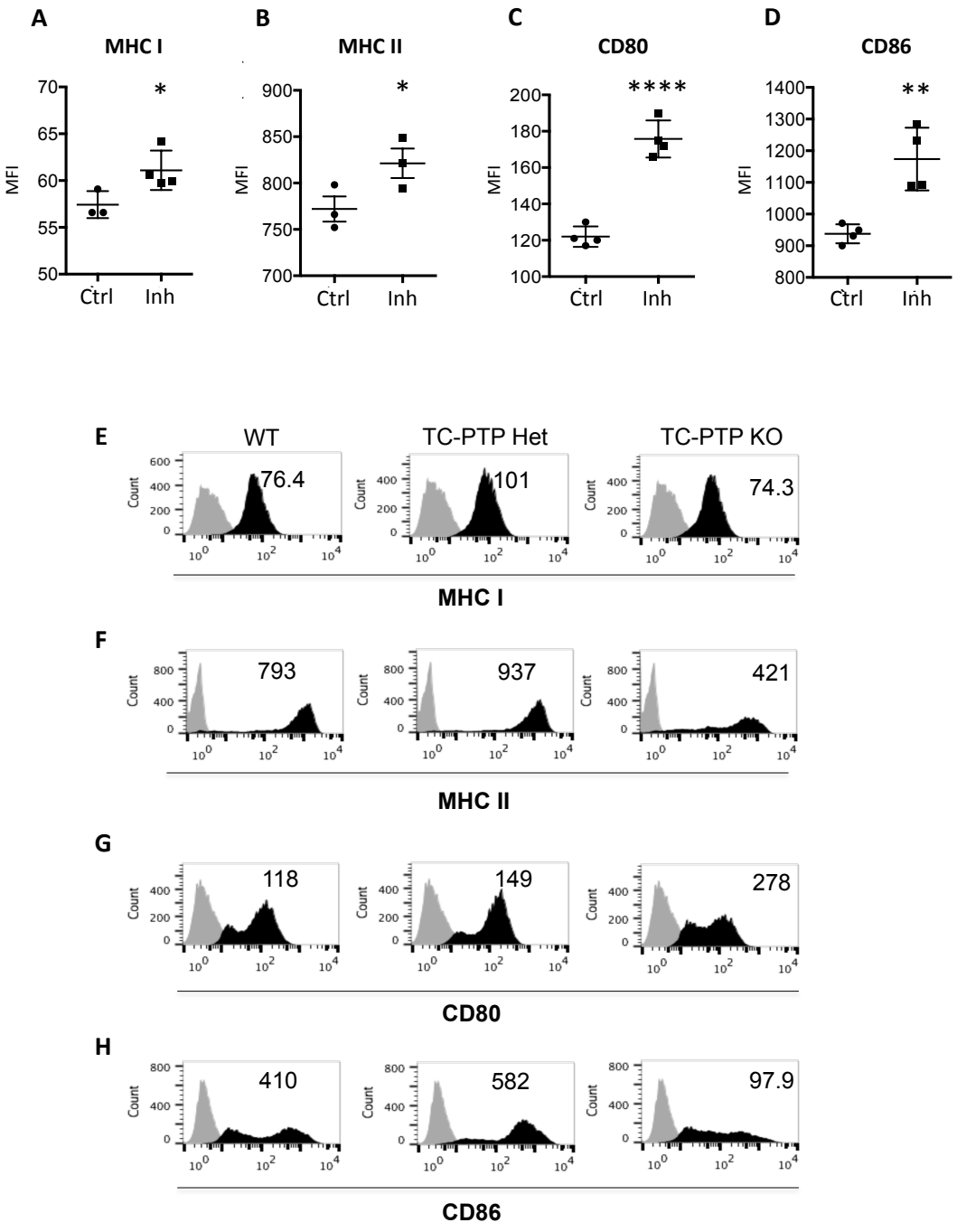


Figure 4.6: Generation of a DC based immunotherapy for use in advanced human pancreatic cancer. Figure legend on next page

Figure 4.6: Generation of a DC based immunotherapy for use in advanced human pancreatic cancer. **(A)** schematic protocol for the use of PTP inhibitor in the generation of highly immunogenic DCs **(B)** Pancreatic cancer patient-derived monocyte DCs were generated in the presence or absence of a PTP inhibitor. Forty-eight hours post activation with MPLA and IFN-γ supernatants were collected and production of IL-12 was measured by ELISA. **(C)** IFN-γ production by activated antigen-specific T cells co-cultured with mature patient-derived DCs with or without PTP inhibitor treatment in an ELISpot assay. CA19-9 and CEA were used as tumor associated antigens to prime autologous patient DCs. Significant differences are represented by asterisk as determined by Mann Whitney test (****P values <0.00005, ***, P < 0.0005, **, P<0.005; *, P<0.05). The results are representative of three independent experiments.



Supplementary Figure 4.7: Phenotype of inhibitor-treated and TC-PTP BMDCs.

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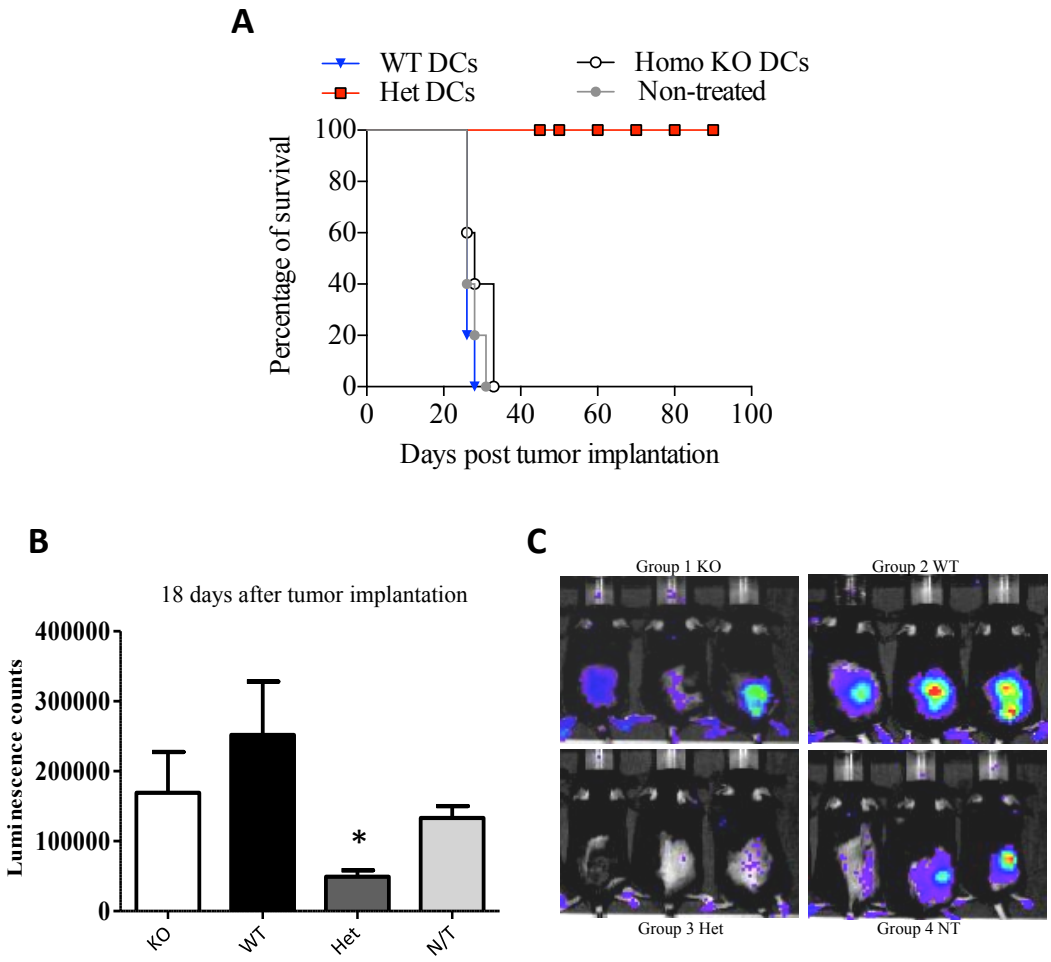
Supplementary Figure 4.7: Phenotype of inhibitor-treated and TC-PTP BMDCs.

Expression of maturation markers on mature DCs treated with inhibitor or derived from TCPTP mice. Mouse BMDCs were prepared as previously described. A-D) MFI of MHC class I, class II, CD80 and CD 86 expression as indicated from DCs treated with inhibitor or non treated controls (n=3-4, results are representative of two independent experiments). E-H) representative FACS plots for MHC class I, class II, CD80 and CD86 as indicated for BMDCs from WT, TC-PTP heterozygous or KO mice (n=4). Significant differences are represented by asterisk as determined by students T test (****P values <0.00005, ***, P < 0.0005, **, P<0.005; *, P<0.05).



Supplementary Figure 4.8: Prophylactic vaccine potential of TC-PTP heterozygous DCs. **(A)** Kaplan-Meier survival analysis of TC-PTP conditional CD11c Cre or WT mice SC injected with $5x10^5$ EG7-OVA lymphomas after being pretreated twice IP with ovalbumin solution (5 mg/mouse) one week prior to tumor challenge. **(B)** *In vivo* bioluminescence measurements of tumor burden at 18 days post tumor implantation. **(C)** Representative images taken on day 18 post tumor implantation showing corresponding tumor burdens. Significant differences are represented by asterisk (*P* values <0.05), n=5.

4.5 Materials and methods

Mouse studies

All animal procedures were carried out using 6-10 week old male C57Bl/6n mice according to the Canadian Council on Animal Care ethical regulations and were approved by the McGill University Research and Ethics Animal committee. PTP1B mice were generated as previously described [148]. *Ptpn1*$^{fl/fl}$ mice were obtained from the laboratory of Dr. Gerard Karsenty [351]. Mice with the floxed TC-PTP allele were generated in our laboratory (Unpublished, Bussières-Marmen S., 2015). Dendritic cell specific deletions were obtained by crossing *Ptpn1*$^{fl/fl}$ or *Ptpn2*$^{fl/fl}$ mice with Cd11c-Cre transgenic mice (The Jackson Laboratory). For the tumor implantation, subcutaneous injections of 5 x 10^5 GFP-luciferase expressing lymphoma cells suspended in 100 µl PBS were subcutaneously injected into syngeneic C57Bl/6n 6-10 week old males as described [350]. Therapeutic intraperitoneal injections of OVA-pulsed DCs derived from *Ptpn1*- *Ptpn2*-CD11cCre mice or treated with PTP inhibitor were performed as indicated in the figure legends. For prophylactic treatments, intraperitoneal injections of OVA solution (5 mg/mouse, Sigma Aldrich) were performed in *Ptpn2*$^{fl/fl}$ - *Ptpn2*$^{wt/fl}$ – or *Ptpn2*$^{wt/wt}$ Cd11c-Cre mice two weeks prior to tumor challenge with 5x10^5 EG7-OVA lymphomas cells. Mice were then monitored for lymphoma development by caliper measurements and/or whole body luminescence imaging (as indicated) using the IVIS 100 in vivo imaging system (PerkinElmer). In vivo tumor cell proliferation was determined by calculating the total flux (photons/second) per animal at the indicated time points.

Generation of murine BMDCs

Murine monocytes were isolated from the bone marrow of PTP1B, TC-PTP or corresponding WT littermates by CD11b positive selection using the EasySep® mouse monocyte CD11b positive selection kit (StemCell technologies). Mouse monocytes were cultured with GM-CSF and IL-4 (20 ng/ml each) (Peprotech) for six days to allow their

differentiation into immature DCs. On day three, half volume of the differentiation media was replaced with new media and cytokines. On day six, immature DCs were stimulated with 1ug/ml LPS (Sigma) and with or without 1mg/ml chicken ovalbumin peptide (Sigma) for an additional 24 hours to induce maturation. On day seven both supernatants and cells were collected. For the generation of PTP-inhibitor treated DCs is as describe above with the addition of 4μM of PTP inhibitor in the culture media during the differentiation and maturation of DCs. (The inhibitor was kindly provided by Brian Kennedy, Merck Frosst, Pointe-Claire, Qc).

Phenotypic analysis of BMDCs

Mature DC cell culture supernatants were tested for the levels of IL-10 and IL-12 using enzyme-linked immunosorbent assay (ELISA) kits (Biolegend). Mature DCs were stained immediately for flow cytometry. The differentiation and maturation status of DCs was determined via labeling with fluorescence-conjugated antibodies specific for MHC class I and class II, CD80 and CD86 (Biolegend). The expression of these cell surface markers was determined by flow cytometry (BD FACScalibur) and analyzed using FlowJo software (TreeStar). To assay T cell activation, DCs were purified as described. LPS +mature and OVA-pulsed DCs derived from WT, Het or KO PTP1B mice were co-cultured with either splenic purified $CD8^+$ or $CD4^+$ T cells isolated from OT-1 or OT-II transgenic mice respectively (Charles river). The cells were co-cultured for 48 hours and the amount of secreted IL-2 was determined in the supernatant.

Generation of a stable luciferase expressing lymphoma cell line

EG7-OVA lymphoma cells (ATCC) were placed in culture and subsequently infected, as described [350], with a murine stem cell virus (MSCV) based IRES-GFP vector (Addgene #19360), subcloned with a firefly luciferase gene (Addgene #18751) into the multi-cloning site using BglII and XhoI restriction sites. Primer sequences were as follows: Luc-BglII GAGAAGATCTACCATGGAAGACGCCAAAAACATAA and Luc-XhoI GAGACTCGAGTTACACGGCGATCTTTCCGCC. GFP positive lymphomas were isolating

using fluorescence activated cell sorting (FACS) (BD FACSaria sorter). Pure populations of GFP-luciferase expressing cells were expanded after sorting and frozen until needed.

PTP inhibitor specificity assay

The hydrolysis of DiFMUP as a substrate for the indicated PTPs was conducted in black 96-well plates (Corning) in a final volume of 100μL at 25°C essentially as described [378]. The reaction was monitored by measuring excitation/emission (358/450) using Varioskan plate reader (Thermo electron). Kinetic measurements were monitored over 10 minutes in 30 seconds intervals and rates were calculated using the slope (relative unit/min).

Generation of mature human DCs

Blood samples were obtained from advanced stage pancreatic cancer patients after providing written informed consent. (Dr. George Zogopolous). Patient DCs were generated from peripheral blood monocytes isolated by positive selection of $CD14^+$ cells (stemcell technologies). Human monocytes were cultured in monocyte attachment media for 1 hour. After one hour, the media was replaced for DC differentiation (StemXVivo serum-free dendritic cell base media) supplemented with GM-CSF (50ng/ml) and IL-4 (35 ng/ml) and PTP inhibitor. Monocytes were cultured for 6 days in the differentiation media. On day three, half volume of the differentiation media was replaced with new media and cytokines. On the day six, the media was replaced with maturation media containing MPLA (2 ug/ml), IFNg (1000 U/ml) and PTP inhibitor (4 uM) [379]. Forty-eight hours post activation with MPLA and IFN-γ, supernatants were collected and analyzed for IL-12 production by ELISA.

ELISpot assay *for IFN-γ Release from Antigen-specific T Cells*

ELISPOT assay for the detection of antigen-specific IFN-γ-producing T cells was performed as described previously [380, 381] and according to the manufactures specifications (R&D systems). Briefly, patient mature DCs cultured in the presence or

absence of PTP inhibitor (4μM) were pulsed with tumor antigens CEA and CA19-9 (2μG/ml, Sigma) and co-cultured with autologous patient T cells previously isolated from peripheral blood using $CD3^+$ positive magnetic bead selection (StemCell technologies). DCs and T cells were co-cultured in a 2:1 ratio ($1x10^5$ T cells: $5x10^4$ DCs) for twenty-four hours. The frequency of IFN-γ producing T cells was quantified using an ELISpot plate reader (BD Biosciences).

Statistical analysis.

Statistical analysis was performed using Prism 6 (Graph-Pad Software). The applied tests are indicated in the figure legends

4.6 Discussion

In the present study we show that modulating the activity of PTP1B or TC-PTP in DCs results in increased expression of key DC maturation markers: MHC class I and class II, costimulatory molecules and production of IL-12. Increased expression of these markers results in an enhanced ability to activate $CD8^+$ T cells which is vital to inducing potent anti-tumor immunity. We demonstrate that therapeutic vaccination of tumor bearing mice with DCs derived from either heterozygous PTP1B or TC-PTP mice is sufficient to significantly delay tumor growth. We were therefore prompted to evaluate the ability of a PTP inhibitor to replicate these findings. Although we determined that the inhibitor also targeted the closely related phosphatase TC-PTP, inhibitor treated DCs had a similar phenotype to those derived from heterozygous mice. Furthermore, DCs treated with the PTP inhibitor successfully delayed tumor growth in a mouse model of lymphoma. To evaluate the potential clinical utility of this approach we treated monocyte derived DCs from advanced stage pancreatic cancer with the PTP inhibitor and demonstrated increased IL-12 production and potent activation of Th1 polarized T cells.

Previously we had demonstrated that complete loss of PTP1B negatively impacted the ability of the immune system to respond to tumor challenge. PTP1B deficient mice were

unable to produce mature and functional DCs resulting in decreases in survival (**Chapter 3**). In lieu of these findings we had evidence to indicate that by modulating the activity of PTP1B either in the heterozygous state where approximately 50% of the enzymes activity remains, or through pharmacological inhibition we could delay tumor growth in mice. The concept of heterozygous advantage is well established and numerous examples can be found throughout the literature [382-385]. Our lab has previously shown that the use of the PTP inhibitor in an ErbB2 model of breast cancer resulted in a significant delay in tumor growth [267]. Furthermore when we initiated crosses of PTP1B mice with Eμ-myc mice, we observed significant delays in tumor onset in mice that were heterozygous for PTP1B (**Appendix I**). While these observations remain to be fully explored it is clear that influencing the activity of PTPs will have dramatic effects on cellular signalling.

Effective anti-tumor therapies require antigen presentation by DCs in the context of Th1 polarizing cytokines. A potent Th1 response will recruit not only T cells but NK cells and macrophages to aid in tumor clearance [386, 387]. Th1 polarization is predominantly effected through robust IL-12 production by DCs and is sufficient to activate potent T and NK cell responses primarily through the stimulation of IFN-γ production [388]. However, in the absence of tumor antigen presentation by DCs, IL-12 administration alone was shown to be sufficient to delay or even prevent tumor growth in both human and animal models [389, 390]. Interestingly, administration of IL-12 in the ErbB2 mouse model of breast cancer resulted in significant delays in tumor onset and reduced tumor multiplicity [391]. We noted significant increases in the secretion of IL-12 in DCs treated with a PTP inhibitor (**Figure 4.2 A**) providing a possible explanation for the delay in tumor onset observed in the ErbB2 mouse model upon administration of the same inhibitor. Signal transduction through the IL-12 receptor is mediated by activation of the PTP1B substrates JAK2, Tyk2 [204] that subsequently phosphorylate the downstream transcription factor STAT4 [392]. Several members of the Jak-STAT family of signalling molecules are substrates of both PTP1B and TC-PTP. It would therefore be beneficial to

analyze JAK-STAT activation in the context of PTP-heterozygous and inhibitor treated DCs to provide a possible mechanism for the enhanced Th1 activation observed.

Our observations demonstrate that the use of a PTP inhibitor was sufficient to significantly enhance the expression of key DC maturation markers and IL-12 cytokine secretion resulting in significantly delayed tumor growth in mice. Based on these findings we were interested in exploring the use of a PTP inhibitor in the development of a novel DC based vaccine formulation. Current DC based immunotherapy strategies generate mature DCs ex vivo through a combination of differentiation and activation cocktails aimed at producing potent IL-12 secreting tumor antigen-specific DCs [4, 6]. The majority of DCs used in clinical trials are generated from either $CD14^+$ monocytic or $CD34^+$ hematopoietic precursors differentiated in the presence of IL-4 and GM-CSF [393]. We therefore employed the same strategy in the generation of mature monocyte-derived DCs from advance stage pancreatic cancer patients. The use of *ex vivo* generated DCs for cancer immunotherapy are advantageous over endogenous DCs as they are more prevalent in peripheral blood and have not encountered the immune suppressive factors produced by the tumor microenvironment [394]. DCs generated from bone marrow precursors were shown to be superior to mature splenic DCs derived from tumor bearing mice at eliciting potent Th1 anti-tumor responses [395].

Upregulation of co-stimulatory molecule expression and DC activation markers during DC maturation are essential to induce effective Th1 cell differentiation and potent anti-tumor responses [6]. Several groups have shown promising findings utilizing DC vaccine approaches that enhance the antigen presentation capacity and induce potent Th1 cytokine production. One strategy being pursued to enhance DC immunotherapy is based on modifications of costimulatory molecules. Electroporation of DCs with mRNA for CD70, CD40-ligand and a constitutively activated TLR4 resulted in increased IL-12 production and potent anti-tumor immunity in a patient with stage IV melanoma [396]. Similarly we demonstrated that a PTP inhibitor significantly enhanced expression of

MHC and costimulatory molecules resulting in increased antigen presentation capacity and Th1 cytokine production. Recently, Takakura et al. showed in a small phase I trial of advanced stage pancreatic cancer patients that DCs pulsed with MHC class I and class II restricted wilms tumor antigens was able to increase IFN-γ production and T cell proliferation [397]. Another study using lethally irradiated tumor cells transduced to secrete IL-12 and fused to DCs showed significant reduction in mouse lung metastasis [398]. These studies highlight some of the different strategies currently being evaluated in DC immunotherapy, they each however share the common goal of increasing production of potent Th1 promoting cytokines and promoting tumor antigen-specific T cell proliferation.

To observe the capacity of our vaccine to induce a Th1 response we measured both IL-12 production by mature DCs and IFN-γ production in an ELISpot assay. We observed significant increases in the production of IL-12 from all three of the patients DCs and increased IFN-γ production from two out of three patients. ELISpot assays are frequently used in during clinical trials to measure tumor antigen-specific Th1 responses in patients [399, 400]. While we determined that PTP inhibitor treated DCs induced significantly more production of the Th1 cytokine IFN-γ this assay does not provide information as to the extent of potential Th2 responses. While generally considered to be detrimental to the development of anti tumor immunity the Th2 response would need to be determined before any clinical implementation of PTP inhibitors could be realized. Th2 responses are characterized as by the secretion of cytokines IL-4, IL-6 and IL-10 and evoke strong antibody mediated responses at the expense of inhibiting potent anti tumor T cell function [401-403]. Th2 cytokines IL-4 and IL-10 are capable of polarized macrophages to the pro-tumor M2 form [404] which have been shown to secrete high amounts of TGF-β and epidermal growth factor leading to pulmonary metastasis of mammary carcinomas [405]. Therefore any novel DC based vaccine would need to polarize T cells towards an increased Th1/Th2 ratio.

In summary we have shown that modulating the activity of PTP1B and TC-PTP in DCs upregulates key markers of DC maturation leading to significant delays in tumor growth. Importantly, DCs derived from Pac patients in the presence of a PTP inhibitor were superior in their ability to activate tumor antigen-specific autologous T cells that could potentially lead to increased tumor cell death. Because this approach is based on the *ex vivo* manipulation of DCs which could be primed with multiple tumor antigens it could be utilized for almost any type of malignancy. We therefore propose to pursue the use of a PTP inhibitor in the development of DC based immunotherapies for the treatment of cancer.

4.7 acknowledgements

The authors would like to thank Ailsa lee loy and Dr. Noriko Uetani for their technical assistance as well as Teri Hatzihristidis and Valerie Vinette for critical reading of the manuscript. This work was supported by a CIHR operating grant M.L. Tremblay (MOP-62887) M. Feldhammer is a recipient of a Canadian Institutes for Health Research (CIHR) Frederick Banting and Charles Best doctoral research award; C. Penafuerte is a recipient of a CHIR post-doctoral fellowship award. M.L. Tremblay is the holder of the Jeanne and Jean-Louis Levesque Chair in Cancer Research.

CHAPTER 5: STAT1 AND STAT4 MODULATE THE PREFERENTIAL MATURATION OF DENDRITIC CELLS BY THE PROTEIN TYROSINE PHOSPHATASES, PTP1B AND TC-PTP

Matthew Feldhammer, Claudia Penafuerte, Nikita Desai, Valerie Vinette and Michel L. Tremblay. *Manuscript in preparation*

5.1 Preface to the manuscript

We have established that by modulating the activity of PTP1B and TC-PTP through the use of a dual specificity inhibitor we could increase the expression of key DC maturation markers. DCs treated with the inhibitor displayed increased secretion of potent Th1 directing cytokines and enhanced the activation of tumor antigen specific T cell responses. A complex signalling network including several transcription factors that are regulated by both PTP1B and TC-PTP controls DC maturation. In light of these promising results we were interested in probing the signalling pathways that were activated upon the modulation of PTP1B and TC-PTP.

5.2 Abstract

Dendritic cells (DCs) are key mediators of the innate and adaptive immune responses. The generation of potent anti-tumor immunity is dependent upon key Th1-promoting signals produced by mature DCs. We have previously demonstrated that by modulating the activity of protein tyrosine phosphatases (PTPs) 1B and TC-PTP we could increase the expression of key DC maturation markers resulting in increased anti-tumor immunity. Here, we interrogated the activation of various members of the janus family of protein tyrosine kinases (JAKs) as well as the signal transducers and activators of transcriptions (STATs) in DCs with altered PTP1B and/or TC-PTP activity. Mouse and human pancreatic cancer patient DCs displayed increased activation of STAT4 upon modulation of PTP activity. This finding was further highlighted by the enhanced production of key Th1-promoting cytokines in DCs treated with a PTP inhibitor. Microarray analysis of DCs revealed global transcriptional differences upon modulation of PTP activity resulting in the upregulation of key genes associated with DC maturation. The current data present a novel mechanistic insight behind the increased expression of DC maturation markers and potent anti-tumor activity observed in DCs with altered PTP activity.

5.3 Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) in the immune system, capable of coordinating both innate and adaptive immunity. DCs can activate a variety of immune responses due to their ability to induce differentiation of naive T cells into a variety of effector cell types such as helper $CD4^+$ T cells (Th), cytotoxic $CD8^+$ T cells (CTLs) and regulatory T cells (Tregs) [330]. The ability of DCs to prime effective anti-tumor responses is hinged upon antigen presentation in the context of robust MHC, and costimulatory molecule expression as well as potent Th1 polarizing cytokine secretion [4, 332, 368]. It is well established that DC functioning is regulated in a maturation-dependent fashion. Immature DCs, while highly capable of capturing antigen in the periphery, are poor activators of T cell differentiation often resulting in immune tolerance, while mature DCs are highly adept at eliciting potent effector cell responses [23, 59, 70, 406].

One of the most critical cytokines produced by mature DCs for the induction of Th1 differentiation is IL-12. Mice deficient for IL-12 displayed compromised natural killer cell functioning and an inability to induce Th1 cell differentiation [407]. Dendritic cells poses a high affinity IL-12 receptor (IL-12R) and subsequently are able to produce IFN-γ in response to IL-12 stimulation. The IL-12/IFN-γ axis potentiates Th1 cell differentiation through the activation of the janus family of protein tyrosine kinases (JAKs), which upon activation will phosphorylate the signal transducers and activators of transcriptions (STATs) [299, 408]. In DCs, IL-12 induces tyrosine phosphorylation and activation of the IL-12R through the activation of the janus kinases JAK2 and TYK2, which, in turn, phosphorylate and activate STAT4 [85, 86]. This pathway is essential for DC activation in an autocrine fashion, as well as IL-12-dependent IFN-γ production by DCs [87]. In addition to activation of STAT4, STAT1 was shown to be required in mature DCs for the production of IL-12 via IFN-γ-initiated signalling [88] as well as the upregulation of the costimulatory molecule CD40 [82]. The two highly related protein tyrosine phosphatases (PTPs) TC-PTP and PTP1B are major negative regulators of the JAK-STAT signalling

pathways. *In vitro* analysis with substrate trapping mutants indicated that PTP1B stably interacts with JAK2 and TYK2 [204] leading to the inhibition of STAT4 activation [409], whereas TC-PTP interacts with JAK1 and JAK3 [410], suggesting that these enzymes may also regulate DC maturation and activation [312, 370].

In this report we generated DCs harbouring deletions of PTP1B, TC-PTP and in parallel, treated DCs with a PTP inhibitor in order to explore the roles of JAK-STAT signalling in their maturation. We show that DCs either heterozygous for PTP1B, TC-PTP or treated with a PTP inhibitor display enhanced STAT1 and STAT4 activation. DCs derived from pancreatic cancer patients in the presence of a PTP inhibitor also displayed dose-dependent increases in STAT4 activation. Microarray analysis of inhibitor-treated DCs highlighted expression of genes associated with DC maturation. We further confirmed the enhanced production of key Th1-promoting cytokines in DCs derived from PTP1B/TC-PTP double heterozygous mice as well as DCs treated with the PTP inhibitor. These findings suggest that modulating the activity of PTP1B and TC-PTP in DCs results in activated JAK-STAT signalling and influences the production of key Th1-promoting cytokines

5.4 Results

5.4.1 Modulation of PTP1B and TC-PTP affects JAK-STAT signalling

Previously we demonstrated that by modulating the activity of PTP1B and TC-PTP we could increase the expression of key maturation markers in DCs derived from both mice and human pancreatic cancer patients (**Chapter 4**). The JAK-STAT signalling pathways play an essential role in the regulation of DC maturation and activation. Both PTP1B and TC-PTP have been established as critical modulators of JAK-STAT signalling and therefore we hypothesized that it was through these pathways that PTP signalling was exerting its preferential activity. In order to investigate this we probed bone marrow-derived DCs (BMDCs) protein lysates from PTP1B (*PTPN1*-CD11cCre), TC-PTP (*PTPN2*-CD11cCre) mice

as well as BMDCs treated with a PTP inhibitor with phosphospecific antibodies to assess the extent of activation of various JAK and STAT molecules.

STAT1 and STAT4 are known to be key positive regulators of DC maturation; knockdown of STAT1 was shown to negatively regulate expression of the key costimulatory molecule CD40 compromising DC maturation [82]. STAT4 activation is induced in a maturation-dependent fashion in DCs and is essential for the production of the key Th1-promoting cytokine IFN-γ [85, 411]. We therefore examined the phosphorylation levels of STAT1 and STAT4 in BMDCs from *PTPN1*-CD11cCre and *PTPN2*-CD11cCre mice as well as BMDCs treated with a PTP inhibitor. We observed increases in the phosphorylation of STAT1 and STAT4 in BMDCs from *PTPN1*$^{fl/wt}$-CD11cCre mice and to a lesser extent in *PTPN2*$^{fl/wt}$-CD11cCre mice when compared with WT and KO littermates (**Figure 5.1 A**). We then investigated if a PTP inhibitor could further enhance the phosphorylation of STAT1 and STAT4 observed in the individual heterozygous BMDCs due to its targeting of both PTP1B and TC-PTP. We observed increases in the amount of STAT1 and STAT4 phosphorylation detected in BMDCs treated with inhibitor when compared to non-treated controls (**Figure 5.1 A**). In addition, the PI3K/Akt pathway can regulate the activation of NF-κB which plays an important role in regulating the transcription of key DC maturation and activation genes [51, 412-415], therefore we interrogated the extent of Akt phosphorylation in *PTPN1*- and *PTPN2*-CD11cCre mice as well as inhibitor-treated BMDCs. We observed increased phosphorylation of Akt in both *PTPN1*$^{fl/wt}$-CD11cCre and *PTPN2*$^{fl/wt}$-CD11cCre BMDCs as well as in inhibitor-treated BMDCs (**Figure 5.2 A**). Hence, in addition to regulating STAT signalling, NF-κB activity may also be preferentially enhanced upon modulation of PTP activity. These results indicate the activation of STAT1, STAT4 and Akt signalling pathways upon modulation of PTP1B and TC-PTP activity in DCs.

Previously, we utilized a PTP inhibitor to increase the ability of pancreatic cancer patient DCs to activate T cells in an ELISpot assay (**Chapter 4 Figure 4.6 C**). We therefore sought

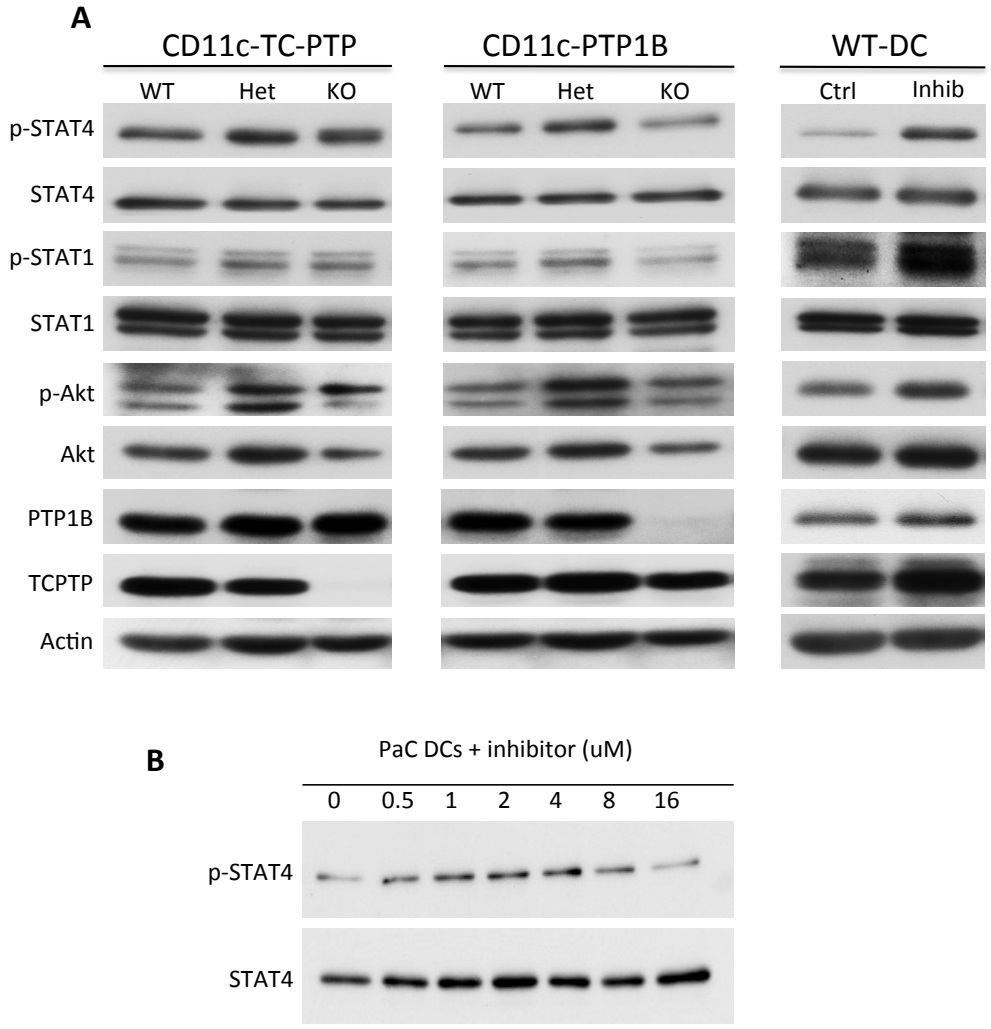


Figure 5.1: Modulation of PTP1B and TC-PTP activities results in increased Stat1, Stat4 and Akt signalling. (A) BMDCs were generated from TC-PTP and PTP1B: WT, Het or KO mice, as well as inhibitor-treated DCs. Twenty-four hours after LPS stimulation cells were harvested, lysed and subjected to SDS-PAGE. Lysates were immunoblotted using phospho-specific and full length antibodies for STAT1, STAT4 and Akt, blots were stripped and re-probed with full length PTP1B, TC-PTP and actin. **(B)** Cell lysates of mature DC cells derived from pancreatic cancer patients blotted with antibodies against phospho-specific and full length Stat4. Experiments were performed in duplicates using a minimum of two biological replicates for PTP1B, TC-PTP and in triplicate for inhibitor treated BMDCs.

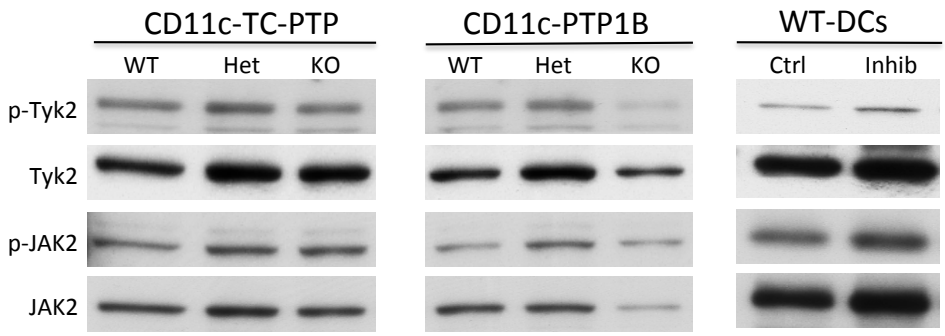


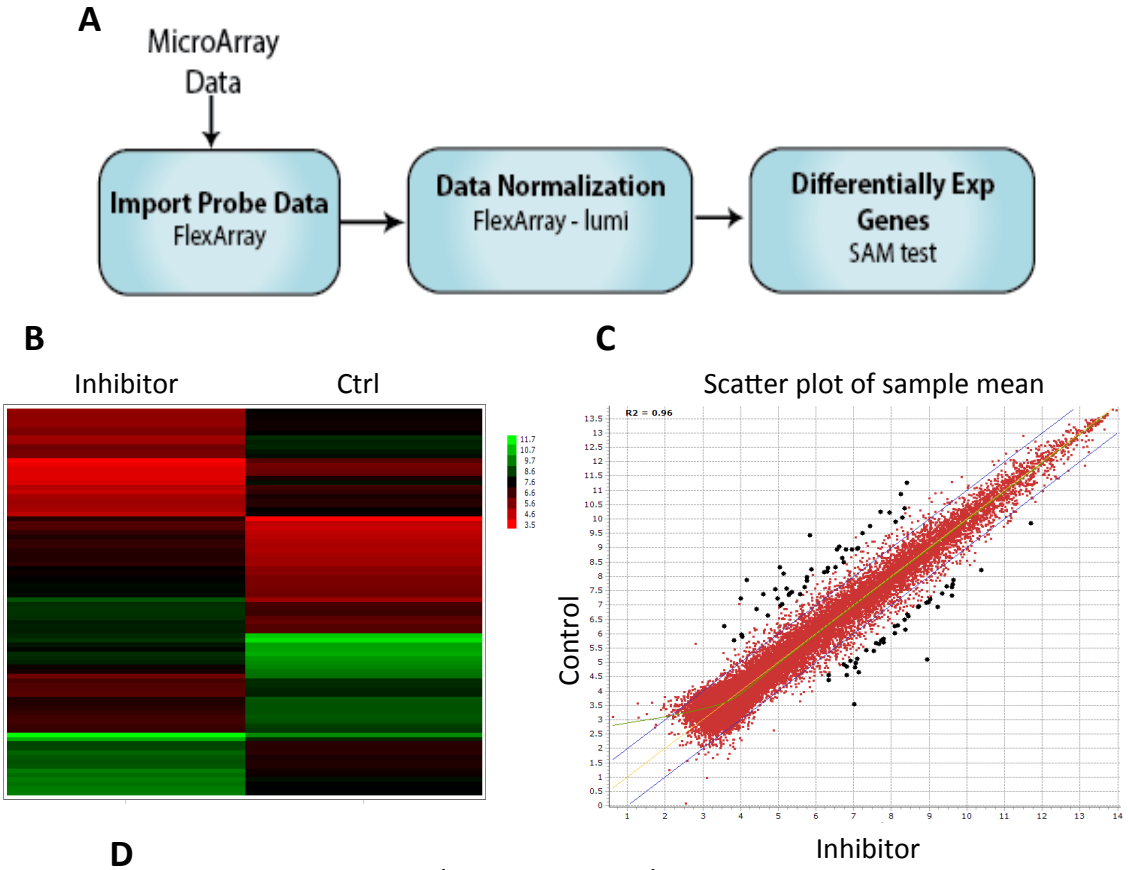
Figure 5.2: Modulation of PTP1B and TC-PTP activities does not affect Jak2 and stat2 signalling. Cell lysates of mature DC cells derived from WT, inhibitor-treated, TC-PTP and PTP1B het and KO mice were prepared as in figure 5.1 and blotted with antibodies against phospho-specific and full length Jak2 and Tyk2. Experiments were performed in duplicates using a minimum of two biological replicates for PTP1B, TC-PTP and in triplicate for inhibitor treated BMDCs.

to determine if this observation correlated with increases in the amount of STAT4 phosphorylation. Pancreatic cancer patient derived DCs displayed a significant dose-dependent increase in the phosphorylation levels of STAT4 (**Figure 5.1 B**). We did however observe a decrease in STAT4 phosphorylation beginning after 8μM possibly due to increased proteasomal degradation following sustained STAT activation or activation of the suppressor of cytokine signalling molecules (SOCS) [2, 416].

We did not observe any overt changes in the phosphorylation levels of TYK2 and JAK2 (Figure 5.1) in the BMDCs from the heterozygous CD11cCre mice when compared with WT or KO. These results suggest that the maturation phenotype observed in heterozygous and inhibitor-treated BMDCs is not due to enhancement in JAK2 or Tyk2 signalling and rather to the downstream transcription factors STAT1, STAT4 and Akt.

5.4.2 Inhibitor-treated BMDCs upregulate genes associated with cellular development, differentiation and immune processes

The STAT family of transcription factors can regulate the expression of a large number of genes involved in a variety of different cellular processes in addition to those implicated in DC maturation. Therefore to explore the global gene expression profiles of DCs treated with a PTP inhibitor, we utilized the Illumina MouseWG-6 microarray. BMDCs were prepared as described in the methods section in presence or absence of 4 μM PTP inhibitor and then subjected to RNA extraction prior to labeling and hybridization. Microarray data was then analyzed using the FlexArray software and differentially expressed genes were identified by the significance analysis of microarray (SAM) test (**Figure 5.3 A**). Analysis of two biological replicates for both inhibitor-treated and non-treated controls reveals significantly different global transcription profiles (**Figure 5.4 B-C**). Using the SAM test we generated a list of 164 genes that were differentially expressed in response to PTP inhibitor treatment (log fold change >1.5 (**Appendix I**)). To extend our understanding of the significance these transcription profiles represented, we analyzed genes that were statistically upregulated in the inhibitor-treated BMDCs in



D

Gene ontology term enrichments

Term	frequency	P-value
positive regulation of biological process (GO:0048518)	153	1.03E-11
positive regulation of immune system process (GO:0002684)	45	1.89E-11
positive regulation of cellular process (GO:0048522)	138	1.07E-10
regulation of developmental process (GO:0050793)	88	2.13E-10
inflammatory response (GO:0006954)	30	2.41E-08
positive regulation of response to stimulus (GO:0048584)	67	9.10E-08
defense response (GO:0006952)	46	5.17E-07
regulation of protein metabolic process (GO:0051246)	76	5.52E-06
positive regulation of cell differentiation (GO:0045597)	42	1.10E-05
positive regulation of cell migration (GO:0030335)	26	1.51E-05
leukocyte chemotaxis (GO:0030595)	13	8.74E-05

Figure 5.3: Inhibitor-treated DCs upregulate genes associated with positive cellular development, differentiation and immune processes. Figure legend on next page

Figure 5.3: Inhibitor-treated DCs upregulate genes associated with positive cellular development, differentiation and immune processes. A) Schematic showing work flow for microarray data acquisition and analysis **(B)** Heatmap visualizing gene expression profiles of differentially expressed genes between inhibitor treated and control BMDCs using two biological replicates. Colors represent high (Green) and low (Red) normalized intensity. **(C)** Scatter plot of sample means between mature WT and PTP inhibitor-treated DCs, differentially expressed transcripts fall outside the best-fit lines. **(D)** Table of top most significantly enriched gene ontology terms related to DC maturation or function detected in genes upregulated upon treatment of DCs with the inhibitor, the number of genes relating to the term and their corresponding P-value.

the context of biological functions. We utilized software available from the Gene Ontology (GO) consortium (www.geneontology.org) [417] to identify gene terms specifically enriched in the inhibitor-treated BMDCs. The GO terms represent a defined vocabulary describing the biological, cellular and molecular functions of each gene. As predicted we observed significant enrichment in GO terms relating to the overall positive regulation of biological, cellular and immune system processes (**Figure 5.3 D**). For each GO term identified, the frequency of corresponding genes appearing as part of that process as well as their representative p-values are listed (**Figure 5.3 D**). We also observed enrichments for GO terms we would expect to see relating to DC functions including: positive regulation of response to stimulus, defense and inflammatory responses, as well as positive regulation of cell migration (**Figure 5.3 D**). These results reveal that BMDCs treated with a PTP inhibitor undergo global expression changes resulting in significantly different transcriptional profiles including ones implicated in DC maturation and function.

5.4.3 Quantitative real-time polymerase chain reaction (qRT-PCR) confirms upregulation of key genes associated with DC maturation.

We were subsequently interested in determining the differences in expression for those genes with known importance for DC maturation and function. By interrogating the microarray data, we observed positive expression profiles as measured by signal intensities for IL-12, TNF-α, PI3K, MyD88, CIITA and the receptor for IFN-γ (**Figure 5.4 A**). In addition, we observed a decrease in the expression levels of the receptor for the inhibitory cytokine IL-10 whose expression is negatively correlated with DC maturation [418]. To confirm the expression of key DC genes produced upon maturation, we performed qRT-PCR. Importantly, we observed significantly elevated expression of the key inflammatory mediator TNF-α (**Figure 5.4 B**) and the key Th1 cytokine IFN-γ (**Figure 5.4 C**). The MHC class II transactivator CIITA was found to be only slightly upregulated both by microarray and qRT-PCR (**Figure 5.4 D**). These results confirm the upregulation

of key genes required for DC maturation and function upon treatment with a PTP inhibitor.

5.4.4 BMDCs from PTP1B/TC-PTP double heterozygous mice behave similarly to those treated with a PTP inhibitor.

Because we previously determined that a PTP inhibitor targeted both PTP1B and the closely related TC-PTP, we generated a novel tissue-specific double heterozygous mouse to model the inhibitor's effects. In order to accomplish this we crossed *PTPN1*$^{fl/wt}$ mice with *PTPN2*$^{fl/wt}$ mice. Offspring that were double heterozygous were then bred with CD11c-Cre transgenic mice to achieve a DC-specific deletion. Mice harboring double heterozygous deletions of PTP1B and TC-PTP in the dendritic cells (*PTPN1*$^{fl/wt}$-*PTPN2*$^{fl/wt}$-CD11cCre) appear to develop normally and display no overt phenotypic defects (unpublished observation). To explore the genetic expression differences we observed in BMDCs treated with a PTP inhibitor, we generated BMDCs from *PTPN1*$^{fl/wt}$-*PTPN2*$^{fl/wt}$-CD11cCre mice and repeated the qRT-PCR analysis. Trending increases were observed for both TNF-α (**Figure 5.5 A**) and IFN-γ (**Figure 5.5 B**), exhibiting similar behavior to the inhibitor-treated BMDCs. Furthermore, we saw no change in the expression of CIITA (**Figure 5.5 C**) as we had previously seen with BMDCs receiving inhibitor treatment. These changes were indicative of key expression differences reported for inhibitor-treated BMDCs but failed to reach statistical significance due to a limited number of available biological replicates (*PTPN1*$^{fl/wt}$-*PTPN2*$^{fl/wt}$-CD11cCre n=2). Still, these data suggest that DCs double heterozygous for PTP1B and TC-PTP behave similarly to inhibitor-treated DCs confirming the inhibitor specificity towards these two phosphatases.

A

	Control	Inhibitor	P value
IL-12	295	583	2.37E-05
TNF-α	936	2635	2.11E-04
PI3K	823	1005	3.32E-02
MyD88	1211	1368	3.05E-04
CIITA	509	596	1.44E-01
IFNγR2	354	446	1.20E-02
IL-10R	1901	931	4.20E-07

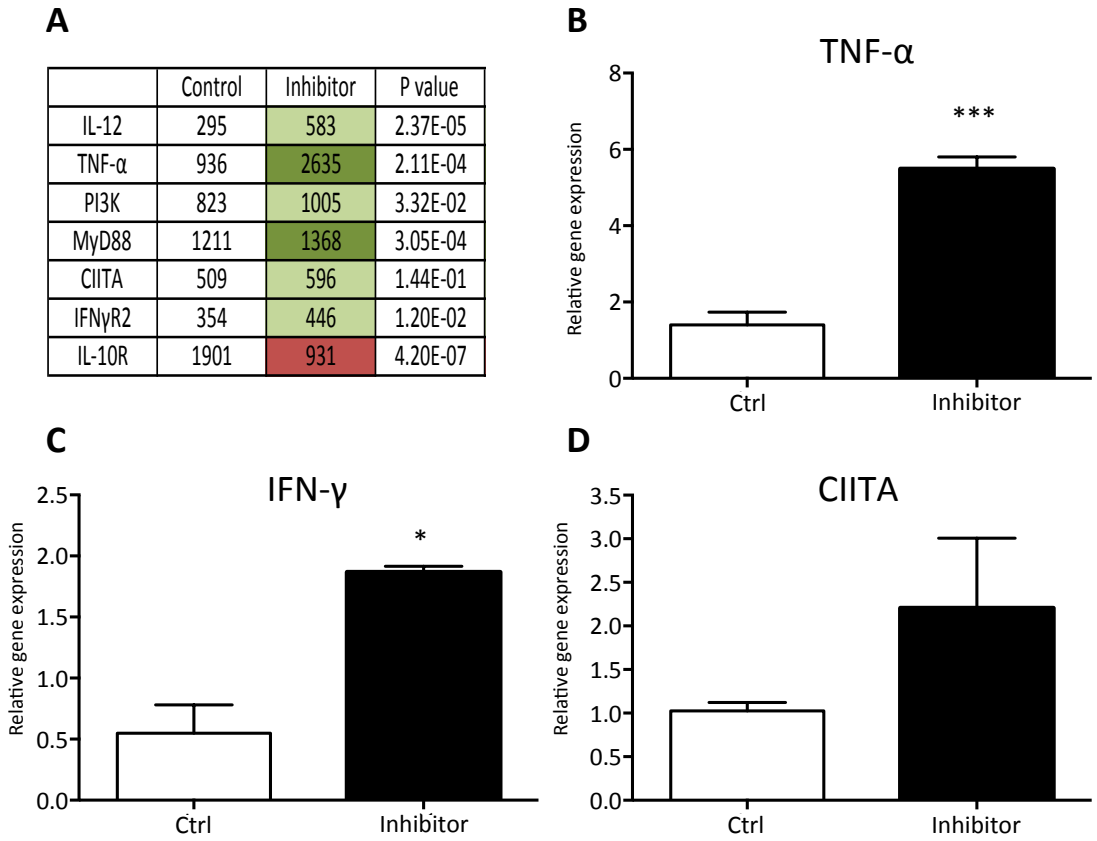


Figure 5.4: qRT-PCR confirms upregulation of key genes associated with DC maturation. (A) Table of probe signal strengths and corresponding P-values for key DC maturation genes significantly up or downregulated in DCs treated with a PTP1B inhibitor. Colors represent high (Green) and low (Red) normalized intensity. Experiments were performed with 2 biological replicates for each control and Inhibitor treated DCs. **B-D)** Total RNA was purified from control and inhibitor treated BMDCs. Isolated RNA was analyzed by qRT-PCR to determine the relative abundance of the indicated genes each normalized to the reference gene Gapdh. Data are means+/- SEM. Significant differences are represented by asterisk according to students T test (*P values <0.05, **P values <0.0005, n=2-6).

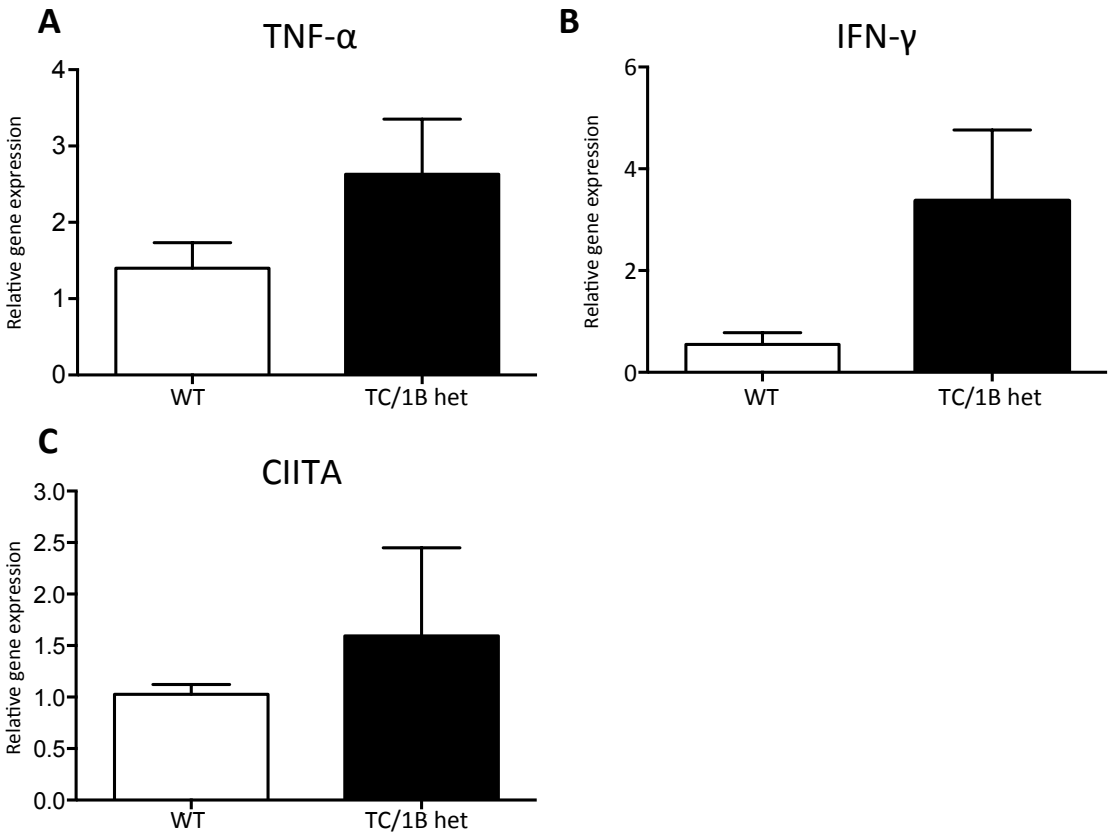


Figure 5.5: BMDCs from PTP1B/TC-PTP double heterozygous mice behave similarly to those treated with a PTP inhibitor A-C) Total RNA was purified from *PTPN1*$^{fl/wt}$-*PTPN2*$^{fl/wt}$-CD11cCre BMDCs. Isolated RNA was analyzed by qRT-PCR to determine the relative abundance of the indicated genes each normalized to the reference gene Gapdh. Data are means+/- SEM. Significant differences are represented by asterisk according to students T test (**P* values <0.05, **P values <0.005, n=2-4).

5.4.5 Significant increases in secretion of key Th1-polarizing cytokines from BMDCs derived from double heterozygous mice, or treated with a PTP inhibitor

Cytokine secretion by DCs is not only a hallmark of maturation but can strongly influence the nature of the subsequent immune response [6, 28]. In order for DCs to prime effective anti-tumor responses from both $CD4^+$ and $CD8^+$ T cells, antigen presentation must take place in the context of the potent Th1-promoting cytokines IL-12 and IFN-γ [53, 325, 419, 420]. Microarray analysis of inhibitor-treated DCs revealed significantly increased expression of IL-12 (**Figure 5.4 A**), while increased production of IFN-γ was detected in both inhibitor-treated and PTP1B/TC-PTP double heterozygous BMDCS by qRT-PCR (**Figures 5.4 C and 5.5 B**). To determine the ability of inhibitor-treated BMDCs and BMDCs from *PTPN1*$^{fl/wt}$-*PTPN2*$^{fl/wt}$-CD11cCre mice to effectively secrete key Th1-promoting cytokines, we collected tissue culture supernatants and determined the amounts of IL-12 and IFN-γ. ELISA analyses revealed that significantly increased production of IFN-γ and IL-12 were present in BMDCS from both inhibitor-treated and *PTPN1*$^{fl/wt}$-*PTPN2*$^{fl/wt}$-CD11cCre mice (**Figure 5.6**). These results indicated that modulating the activity of PTP1B and TC-PTP significantly enhances the secretion of key Th1 cytokines by BMDCs.

Taken together, these results suggest that the preferential maturation of inhibitor-treated as well as heterozygous PTP1B and TC-PTP BMDCs is mediated through enhanced STAT1 and STAT4 molecule signalling, resulting in increased activation of key Th1 cytokines IL-12 and IFN-γ.

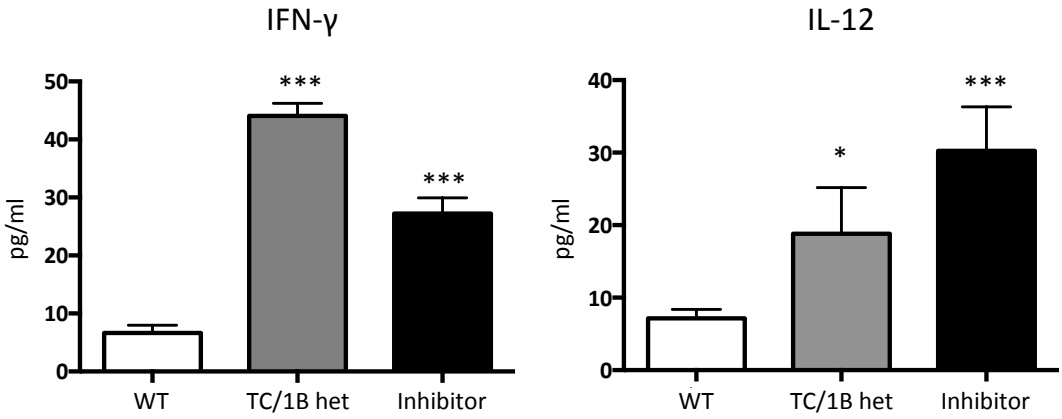


Figure 5.6: PTP inhibitor or double het PTP1B/TC-PTP DCs produce increased amounts of key Th1 polarizing cytokines. BMDCs were generated from inhibitor treated and non-treated controls as well as *PTPN1*$^{fl/wt}$-*PTPN2*$^{fl/wt}$-CD11cCre mice. Twenty-four hours after LPS stimulation supernatants were harvested and tested for the production of A) IFN-γ and B) Il-12. Data are means+/- SEM. Significant differences are represented by asterisk according to students T test (**P* values <0.05, ***P values <0.0005, n=2-4).

5.5 Discussion

Dendritic cells are capable of secreting high levels of cytokines, Il-12 and IFN-γ in the activation of potent Th1 responses required for anti tumor immunity [48, 421]. In addition to cytokine production, DCs provide the first two signals required to activate naïve T cells in the form of antigen presentation coupled to MHC molecules as well as costimulatory molecule interaction [59]. Together these three signals are hallmarks of mature dendritic cells, which are capable of activating T cells and inducing potent anti tumor immunity. We have previously demonstrated that modulating the activity of two protein tyrosine phosphatases (PTP1B and TC-PTP) through genetic or pharmacological means resulted in increased expression of key DC maturation markers leading to enhanced anti tumor activity (**Chapter 4**). In the present study we uncovered a possible mechanism to explain these observations and further characterized some of the changes that occur leading to the production of potent Th1 polarizing cytokines Il-12 and IFN-γ.

Both PTP1B and TC-PTP are major regulators of the JAK-STAT signalling pathways, which are essential in the maturation of DCs. Particularly STAT1 and STAT4 have been shown to be required in the upregulation of genes vital for DC maturation and production of Th1 polarizing cytokines. Mouse studies demonstrated that STAT1 phosphorylation was induced in a maturation dependent fashion and that DCs deficient for STAT1 were impaired in their ability to upregulate the costimulatory molecule CD40 [82]. Interestingly, in immature DCs, STAT1 inhibits the expression of costimulatory molecule CD86. In contrast, mature DCs where STAT1 is activated, display significant upregulation of costimulatory molecule expression upon LPS [82]. A more recent study demonstrated that in addition to low CD40 expression, STAT1 deficient DCs failed to express sufficient levels of MHC class II, CD80 and CD86 to effectively prime Th1 cell responses [422]. The activation of STAT1 is induced by GM-CSF and LPS, which are routinely used to produce mature DCs in cell culture [82]. Similarly we observed STAT1 activation in mature DCs, but this phosphorylation was increased upon treatment with the PTP inhibitor which

was previously shown to result in the upregulation of MHC and costimulatory molecules (**Chapter 4**).

PTP1B has so far not been shown to directly dephosphorylate STAT1, however it was reported to influence the LPS induced activation of TYK2, which is the upstream activator of STAT1 in macrophages [251]. Both TYK2 and JAK2 were previously established as direct substrates of PTP1B [204] so it was surprising that we did not observe any significant differences in the levels of TYK2 or JAK2 activation upon PTP inhibitor treatment (Figure 5.1). This however may be due to differing substrate affinity among different cell types. These previous studies were conducted in full body knockout out animals and the prolonged absence of PTP1B may result in different intracellular signalling responses than we observed upon the relatively acute treatments with the PTP inhibitor. Additionally, using a PTP inhibitor in the generation of DCs may allow the opportunity for other phosphatases to compensate for a short term loss of PTP1B or TC-PTP activity [423]. In contrast to PTP1B, TC-PTP was demonstrated to dephosphorylate STAT1 upon activation with IFN-γ [424] and therefore may play a larger role in contributing to the observed activation of STAT1 in PTP inhibitor treated DCs. STAT1 drives expression of a variety of signalling molecules including the transcription factor T-bet which in turn positively regulates the expression of the IL-12 receptor on the surface of DCs allowing for an increased ability to respond to IL-12 signalling [425, 426].

Similar to STAT1, STAT4 signalling in DCs is also induced in a maturation-dependent fashion [85]. Deletion of STAT4 in DCs and macrophages resulted in an impaired ability to secrete IFN-γ. The TLR ligand, CD40L and cytokines such as IFN-γ are strong inducers of IL-12 production by DCs [87, 427]. IL-12 signalling induces tyrosine phosphorylation and activation of the IL-12 receptor through the activation of JAK2 and TYK2, which, in turn, phosphorylate and activate STAT4 [85, 86]. This pathway is essential for DC activation in an autocrine manner, as well as IL-12-dependent IFN-γ production by DCs [428, 429]. Similarly we observed increased secretion of IL-12 and IFN-γ upon treatment

of DCs with a PTP inhibitor. It is therefore possible to ascribe this increased Th1 cytokine production to the observed increase in STAT4 activation. Since Il-12 secretion can activate STAT4 signalling in DCs in an autocrine fashion however, we cannot rule out the possibility that increased receptor signalling as opposed to decreased PTP activity as being a driving force behind STAT4 activation. Interestingly though as previously mentioned we did not observe any increases in the activation of TYK2 and JAK2 upon treatment of DCs with a PTP inhibitor strongly suggesting that the observed increases were in fact due to a lack of dephosphorylation on STAT4 as opposed to increased upstream signalling by TYK2 and JAK2.

The STAT family of transcription factors can mediate cytokine receptor signalling in both homo- and heterodimeric forms providing another mechanism for the modification of downstream signalling [430]. Interestingly, although IL-12 signalling functions mainly through STAT4 homodimers [431], STAT1 is capable of forming heterodimers with STAT2 upon activation by IFN-γ leading to the regulation of distinct subset of interferon stimulated genes [432]. It would therefore be of interest to analyze the activation dynamics of all members of the STAT family upon treatment of DCs with a PTP inhibitor and interrogate the ratios of both homo and heterodimers of each that are formed.

LPS can mediate DC activation by binding to TLR4, which is coupled to the adaptor molecule MyD88 and leads to activation of NF-κB resulting in expression of costimulatory and MHC molecules as well as the production of Il-12 [50, 413]. Several other pathways have also been reported to play a role in the activation of NF-κB [433]. In the absence of activation signals, NF-κB is sequestered in the cytoplasm and is complexed with the inhibitory protein IκBα [434]. Interestingly, TNF-α can induce DC maturation through the destruction of IκBα, which is a negative regulator of NF-κB [330, 435, 436]. Ritter et al. demonstrated that bone marrow monocytes from TNF-α deficient mice were unable to differentiate into mature DCs [437]. By using a PTP inhibitor to enhance DC maturation through STAT1 and STAT4 hyperactivation, we also observed

increased TNF-α expression, which potentiates the DC immunogenic phenotype through activation of the NF-kB pathway. Although it is currently unclear how PTP1B may regulate TNF-α, TC-PTP was shown to negatively regulate TNF-α expression [288]. Therefore the increased expression of key DC maturation markers observed in PTP inhibitor treated DCs may be regulated through enhanced NF-κB activation downstream of TNF-α, in combination with STAT1 and STAT4.

In summary we have identified a potential mechanism to explain how modulation of TC-PTP and PTP1B activity contributes to the increased expression of key DC maturation markers. DCs treated with a PTP inhibitor displayed hyperactivation of transcription factors STAT1 and STAT4 and increased expression of TNF-α, which correlated with the increased production of potent Th1 promoting cytokines IL-12 and IFN-γ. These findings further support the development of PTP inhibitors to enhance the immunogenic potential of DC based anti cancer vaccines.

5.6 Materials and methods

Mouse studies

All animal procedures were carried out using 6-10 week old male C57Bl/6n mice according to the Canadian Council on Animal Care ethical regulations and were approved by the McGill University Research and Ethics Animal committee. *Ptpn1*$^{fl/fl}$ mice were obtained from the laboratory of Dr. Gerard Karsenty [351]. Mice with the floxed TC-PTP allele were generated in our laboratory (Unpublished, Bussières-Marmen S., 2015). Mice with double heterozygous deletions of both PTP1B and TC-PTP were generated by crossing *Ptpn1*$^{wt/fl}$ mice with *Ptpn2*$^{wt/fl}$ mice. Dendritic cell specific deletions were obtained by crossing *Ptpn1*^{fl}, *Ptpn2*^{fl}, *or Ptpn1/ptpn2*^{fl} mice with Cd11c-Cre transgenic mice (The Jackson Laboratory).

Generation of murine BMDCs

Murine monocytes were isolated from the bone marrow of PTP1B, TC-PTP, PTP1B/TC-PTP or corresponding WT littermates by CD11b positive selection using the EasySep® mouse monocyte CD11b positive selection kit (StemCell technologies). Mouse monocytes were cultured with GM-CSF and IL-4 (20 ng/ml each, Peprotech) for six days to allow their differentiation into immature DCs. On day three, half volume of the differentiation media was replaced with new media and cytokines. On day six, immature DCs were stimulated with 1ug/ml LPS (Sigma) for an additional 24 hours to induce maturation. On day seven, both supernatants and cells were collected and stored until use. The generation of inhibitor treated DCs is as describe above with the addition of 4μM PTP inhibitor in the culture media during the differentiation and maturation of DCs (Inhibitor was kindly provided by Brian Kennedy, Merck Frosst, Pointe-Claire, Qc).

Western blot analysis

For western blot analyses, whole BMDCs were lysed in radioimmunoprecipitation assay buffer. Protein samples were resolved on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblotting. Phospho-specific and total Jak2, Tyk2, STAT1, STAT4, and Akt proteins were detected using polyclonal antibodies (Cell Signalling Technology). β-Actin (Sigma) was used as a loading control and PTP1B (BD) or TC-PTP (Cell Signalling) was detected with a monoclonal antibody (BD). Primary antibodies were followed by horseradish peroxidase-conjugated goat anti–rabbit or mouse IgG (Jackson ImmunoResearch Laboratories). Blots were revealed using Western lightning chemiluminescent substrate (Perkin Elmer).

RNA preparation and microarray analysis

BMDCs were generated with or without a 4μM PTP inhibitor as described above. RNA was isolated using Trizol (Life Technologies) according to the manufacturers recommendations and stored prior to use for either qRT-PCR or microarray analysis. RNA integrity was evaluated with Agilent 2100 BioAnalyzer microfluidics-based platform

(Agilent Technologies). RNA samples were subsequently processed to yield biotinylated cRNA for hybridization to an Illumina MouseWG-6 microarray. The expression data were obtained and imported into the FlexArray (version 1.6.1, Genome Quebec, McGill University) software package for data quality control and statistical analysis of the microarray data. To extract differentially expressed genes as well as expression data for genes of interest, the FlexArray software was used. The Illumina sample probe data for the microarray samples were imported into the software along with the control probe report. Once imported, the lumi algorithm was used to for a robust spline normalization using negative controls for background correction. A two sample Bayesian *t*-test was used to determine which of the genes were significantly differentially expressed. Finally, the following filters were used to filter genes that were considered to be significantly differentially expressed genes: a p-value of 0.05, a T statistic of +/- 2.5 and a log-fold change of +/- 1.5. For gene ontology analysis, ranked gene lists were first generated and then imported into the gene ontology web database (Gene Ontology Consortium).

Quantitative RT-PCR and cytokine analysis.

Total RNA was extracted from BMDCs with the TRIzol reagent (Invitrogen) according to the manufacturers instructions. Any potential contaminating genomic DNA was degraded with the DNase I RiboPure kit (Life Technologies). RNA was transcribed to cDNA with the SuperScript III Reverse Transcriptase Kit (Life Technologies) according to the manufacturers instructions, and qRT-PCR was performed on a LightCycler 480 with SYBR Green Master Mix. Gapdh was used as a reference gene to calculate the relative abundance of the indicated genes of interest. Primers were designed using the Harvard Primer Bank (http://pga.mgh.harvard.edu/primerbank/). Primer sequences are as follows: CIITA forward TAATCTACCACGGTGAGATGCC; CIITA reverse CGGGGAGACTGGGGATACT, TNF-α forward CTGTGAAGGGAATGGGTGTT; TNF-α reverse CAGGGAAGAATCTGGAAAGGTC, IFN-γ forward TGAGACAATGAACGCTAC; IFN-γ reverse TTCCACATCTATGCCACT.

To measure cytokine production, mature inhibitor treated, double heterozygous and control BMDC cell culture supernatants were tested for the levels of IL-12 and IFN-γ using enzyme-linked immunosorbent assay kits according to the manufacturers specifications (Biolegend).

Statistical analysis.

Statistical analysis was performed using Prism 6 (Graph-Pad Software), The applied tests are indicated in the figure legends

5.7 Acknowledgements

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CHAPTER 6: GENERAL DISCUSSION

6.1 PTP1B: From metabolism to immunology

Initially, PTP1B received significant attention in both academia and the pharmaceutical industry when our laboratory demonstrated that PTP1B deficient mice failed to develop type-2 diabetes upon being fed a high fat diet [148]. This study and subsequently many others detailed a prominent role for PTP1B in controlling metabolism by regulating both insulin and leptin receptor signalling [140, 312]. Now, fifteen years after this seminal discovery, despite the fact that there is currently no PTP1B inhibitor in clinical trials, our understandings of PTP1B functions have increased significantly. Early studies on PTP1B focused on the identification of novel targets and expanded our understanding of how it regulates metabolic signalling [147, 204, 281, 371]. Further examination into how PTP1B controls cellular signalling led to its implication in a number of cancers [181, 266, 269, 311]. It is now commonly accepted that, by virtue of the number of diverse signalling pathways under its control, PTP1B can exert both tumor promoting and tumor suppressing roles [140, 262]. More recently, the research focus has shifted towards exploring how PTP1B can regulate immune cell signalling [282, 312, 355, 438]. Three key studies highlight the vital role of PTP1B in the immune system: 1) The identification of the key immune signalling molecules, JAK2 and TYK2 as PTP1B substrates [204]. 2) The discovery that PTP1B deficiency leads to a block in B cell development in a mouse cancer model [266], and 3) The recognition of PTP1B as a negative regulator of macrophage development and activation [282]. These studies prompted us to ask the following questions: How would PTP1B deficiency affect the immune response to cancer? Which immune cells are altered upon PTP1B deficiency? Lastly, which substrates in immune cells could be targeted by PTP1B?

In chapter 2, we addressed these questions by challenging PTP1B deficient mice with B cell lymphomas derived from the transgenic Eμ-myc mouse. We observed significant decreases in overall survival in mice lacking PTP1B, which correlated with increasing numbers of immature, and tolerogenic DCs detected in tumor infiltrates. In addition, we also detected increasing numbers of immune suppressive cell types. Our findings

suggest that in a PTP1B deficient state, tumor cells can proliferate in part due to a suppressed immune response. Interestingly, a recent study by Zhang et al. also reported increased numbers of suppressive immune cells present in PTP1B deficient mice in a DSS-induced colitis model [334]. These results highlight the importance of PTP1B in maintaining the proper coordination of the immune response.

Few reports that detail how the immune system is affected in PTP1B deficient mice are currently available; therefore we utilized a cytokine array to address this issue. We hypothesized that we should observe increases in only Th2 promoting cytokines such as IL-4, IL-6 and IL-10, which are generally associated with decreased cell based immunity and increased cancer progression [322, 323]. This however was not the case as we observed dramatic increases in both Th2 and Th1 cytokines in PTP1B deficient animals. It would therefore be beneficial to repeat the cytokine array post tumor challenge to determine exactly how the immune system is contributing to the increased tumor proliferation we observed. Although this subject is now beginning to be explored, the majority of immunological studies on PTP1B highlight roles in macrophages and B cells, and there are currently no reports, other than those presented here, that explore PTP1B function in DCs.

DCs, as detailed previously, are the sentinels of the immune system, capable of activating potent antigen-specific T cell responses. Because we observed defects in DC maturation upon PTP1B deletion we characterized the ability of DCs from PTP1B knockout mice to activate $CD4^+$ or $CD8^+$ T cells. While we demonstrated that DCs from PTP1B KO mice displayed a decreased ability to activate $CD4^+$ T cells we did not fully characterize the T cell responses in terms of Th1 and Th2 polarization. As detailed in the introduction, DCs are capable of producing potent Th1 and Th2 polarizing cytokines which when coupled with antigen presentation in the context of MHC and costimulatory molecules provide the necessary signals to activate the adaptive immune response [23, 70]. Therefore, it would be of interest to assess the amounts of both IFN-γ and lL-4 as

the main Th1 and Th2 cytokines being produced upon T cell activation in order to fully characterized the state of T cell polarization.

Based on previous reports highlighting an important role for PTP1B in regulating macrophage development and function, it was surprising that we observed no differences in the numbers of macrophages infiltrating the tumors [282, 318]. Macrophages are often recruited to tumor sites where they facilitate angiogenesis, as well as tumor proliferation and migration, and are correlated with poor patient prognosis [439]. Because we noted no differences in the overall number of macrophages, we did not pursue their investigation further. However, like DCs, which can exist in two functionally distinct maturation states, macrophages can be polarized into either an M1 or an M2 form [440]. M2 macrophages like Th2 cells are generally considered to promote cell growth and suppress the immune response and are therefore utilized by tumors to enhance their proliferation [441]. It would therefore be of interest to interrogate the nature of the macrophages present in the tumor infiltrates to determine if the ratios of M1:M2 are the same between $PTP1B^{+/+}$ and $PTP1B^{-/-}$ mice. This could be done using flow cytometry on isolated macrophages and staining for the commonly used surface markers CCR7 and CD163 for M1 and M2 respectively [442].

PTP1B has been extensively characterized with respect to the diverse number of substrates it regulates. A large-scale proteomic analysis by Mertins et al. identified 124 proteins that underwent changes in tyrosine phosphorylation upon deletion of PTP1B [147]. Although this number does not reflect the actual amount of bona fide direct substrates, it serves to highlight the ability of PTP1B to influence the global cellular signalling networks of the cell. Due to the rapid enzymatic nature of the interaction between PTPs and their substrates, it is necessary to mutate the PTP active site in order to capture and identify substrates. The use of substrate trapping mutants therefore is often employed in the identification of direct PTP targets [209]. Our lab and others have previously utilized these approaches to identify several members of the JAK-STAT family

as direct PTP1B substrates, including Jak2, Tyk2, STAT3 and STAT6 [204, 281, 319]. Interestingly, STAT5 had also previously been identified as a substrate of PTP1B downstream of activation by the milk hormone prolactin, however this article has since been retracted [319]. Of all the members of the STAT family, STAT3 and STAT5 are most often implicated in the progression of cancer and are therefore being pursued as pharmacological targets [89, 443, 444]. STAT3 and STAT5 have also been implicated in the negative regulation of DC maturation, which prompted our investigation into their activation state [84, 90, 327]. Not surprisingly, we observed increased phosphorylation of these two transcription factors, which negatively impacted the ability of DCs to mature. Several other STATs, including STAT1 and STAT4 contribute to DC maturation but, in contrast to STAT3 and STAT5, promote the expression of key DC maturation markers [82, 83, 85]. It would therefore be informative to interrogate the phosphorylation levels of these transcription factors to determine if their activation was inhibited or stimulated upon PTP1B deletion. Presumably, because STAT1 and STAT4 promote DC maturation, they would exhibit either no change or a decrease with respect to their phosphorylation upon PTP1B deletion. Because neither STAT1 nor STAT4 has previously been identified as a direct PTP1B substrate, this analysis should also include the extent of activation of their upstream regulators and their downstream targets in order to form a more informative analysis of how their signalling contributes towards DC maturation in the context of PTP1B deletion.

6.2 PTP1B inhibition: a novel method of harnessing the immune system to fight cancer.

The idea of modifying tyrosine phosphorylation signals in order fight cancer is not an original one. Aberrant receptor tyrosine kinase (RTK) signalling is often associated with cellular processes that lead to tumor cell proliferation and survival [445]. Perhaps the most salient example is the epidermal growth factor receptor (EGFR), which is amplified in over 30% of invasive breast cancers and is strongly correlated with patient survival and time to relapse [446]. The clinical use of RTK inhibitors, which interfere with phosphorylation signalling, such as the EGFR targeted antibody Herceptin, is now

widespread. EGFR activation triggers phosphorylation and activation of a number of downstream signalling molecules such as Ras, PI3K and STAT3 leading to uncontrolled cell growth[447]. Intuitively then, the use PTP inhibitors would promote aberrant RTK signalling further enhancing their oncogenic properties by removing the negative regulatory mechanisms [140, 262]. However, despite potentially enhanced oncogenic signalling, PTP1B deficient mice do not develop spontaneous tumors [266]. This may be due to the existence of functional redundancies, such as other closely related PTPs with overlapping substrate profiles, or PTP1B may differ in the regulation of RTKs with respect to different cell types. In addition, PTP1B has been observed to exert both tumor promoting and tumor suppressing roles in different cancer types [262, 312]. Therefore the use of PTP inhibitors for the treatment of cancer must not be ruled out strictly based on the idea that PTPs provide the 'off' switch to the aberrant RTK signalling. To this end, our lab previously demonstrated that in an ErbB2 model of breast cancer, PTP1B deficiency resulted in increased $p62^{DOK}$ activation, which in turn binds to p120RasGAP and inhibits activation of MAPK signalling leading to a significant delay in tumorigenesis [267]. In addition, our lab has also described a role for PTP1B in promoting prostate cancer cell migration and invasion, tumorigenic properties that were ablated upon PTP1B inhibition [181]. These findings underscore the concept that PTPs can negatively regulate inhibitory signals, and therefore the use of PTP inhibitors could be beneficial in the termination of aberrant signal transduction.

We explored PTP1B deletion as it relates to the regulation of the immune response to cancer in chapters 3 and 4. As detailed, we determined that loss of PTP1B significantly impacted the ability of DCs to properly coordinate the immune response upon tumor challenge. Based on our previous findings, we hypothesized that modulation and not deletion of PTP1B activity may result in the activation of different signalling pathways resulting in increased immune cell activation. We therefore explored the ability of a PTP inhibitor to impact the expression of key DC maturation markers. We noted that in contrast to complete PTP1B depletion, DCs that were either heterozygous for PTP1B or

treated with a PTP inhibitor significantly upregulated the expression of MHC and costimulatory molecules as well as secretion of the key Th1 promoting cytokines lL-12 and IFN-γ which are vital to the induction of anti-tumor immunity.

DCs are the professional antigen presenting cells of the immune system. However, they do not posses effector functions, which are required for killing tumor cells. Therefore, any potential benefits that upregulation of DC maturation markers can confer must be coupled with increased T cell activation and function. To address this, we used both co-culture as well as ELISpot assays to determine the ability of PTP1B heterozygous and inhibitor treated DCs to activate T cells. $CD8^+$ T cells, once activated by DCs, are capable of lysing tumor cells using a combination of cytolytic enzymes [18]. In the co-culture assay, we measured increased $CD8^+$ T cell activation upon incubation with DCs derived from PTP1B heterozygous mice. This result is informative as to the ability of PTP1B heterozygous DCs to promote the increased activation of T cells, however it does not provide insight as to the polarization or cytolytic potential of the resulting T cell response. Based on the increased secretion of the key Th1 cytokine IL-12 by PTP1B heterozygous DCs, it is possible to infer that the T cell activation should be Th1 polarized. However, it would be of interest to directly probe the amounts of IFN-γ and IL-4 being produced by the T cells in order to determine their exact polarization. In addition to examining the extent of T cell polarization, cytolytic effector function can be measured by chromium release assays to determine the ability of activated antigen-specific T cells to secrete cytolytic enzymes.

ELISpot assays are particularly informative in measuring the state of antigen-specific T cell activation and are frequently used in immunotherapy clinical trials [448]. Clinical trials have shown significant positive correlations between the amount of antigen-specific IFN-γ production by T cells as measured by ELISpot and patient survival in melanoma, prostate and breast cancer studies [449-451]. We therefore employed the ELISpot assay in order to determine the ability of patient derived inhibitor treated DCs

to activate antigen-specific T cell responses. Our results support the use of PTP inhibitors to increase the immunogenicity of DC based anti-cancer vaccines.

6.3 PTP1B inhibitors: how to wield a potential double-edged sword.

Despite the intense pursuit, and clinical utilization of RTK inhibitors in cancer therapy, there are currently no PTP1B inhibitors being pursued in clinical trials. Two major concerns, both of which relate to specificity, underlie this lack of progress. First, the highly conserved nature of the active site of PTP1B indicates that specificity may be difficult to obtain. Second, because PTP1B may simultaneously regulate multiple signalling pathways, inhibition may give rise to unwanted side effects [452]. Therefore, it was not surprising that we unveiled that the PTP1B inhibitor utilized in our studies also targeted the closely related enzyme TC-PTP. PTP1B and TC-PTP share ~75% sequence homology in their active sites but differ in regards to their localization and substrate profiles [371, 453]. PTP1B knockout mice display no overt pathologies. In contrast, TC-PTP deficient mice develop systemic inflammation and die 3-5 weeks after birth [288, 454]. In order to pursue the clinical use of a PTP inhibitor that targets both PTP1B and TC-PTP, it is vital to understand the signalling pathways that are affected in order to prevent any potential adverse effects.

Our laboratory had previously uncovered some insight into the effects of modulating both PTP1B and TC-PTP activity by generating double deficient, as well as double heterozygous mutant mice [289]. Mice that were heterozygous for both PTP1B and TC-PTP developed normally. In contrast, deletion of both PTPs resulted in embryonic lethality. T cells from TC-PTP$^{+/-}$-PTP1B$^{-/-}$ mice displayed increased IFN-γ signalling and hyperphosphorylation of STAT1 compared with their littermates, suggesting that these PTPs play non-redundant roles in regulating IFN-γ signalling. These results revealed that only one copy of either TC-PTP or PTP1B was sufficient for normal embryonic development and that modulation of PTP activity can result in beneficial immune signalling provided sufficient amounts of at least one enzyme remains active.

We observed similar results in our DCs with regards to STAT1 activation and IFN-γ secretion upon the use of our PTP inhibitor. We further examined the signalling pathways that were implicated in the increased expression of key DC maturation markers, and uncovered hyperphosphorylation of STAT4 and Akt molecules upon PTP inhibitor treatment. While promoting STAT4 and Akt signalling in DCs is beneficial to the expression of genes required for maturation and T cell activation, hyperactive Akt signalling in other cell types is frequently associated with cancer [455]. Although Akt is unlikely a direct substrate of either PTP1B or TC-PTP, as it is activated as a result of serine and threonine phosphorylation, several upstream activators including the p85 subunit of phosphatidylinositol 3-kinase (PI3K) do undergo tyrosine phosphorylation [456]. To this point, several studies have demonstrated increased PI3K/Akt signalling in PTP1B deficient cells [281, 317, 355]. These studies demonstrate how PTP1B inhibition can be a double-edged sword in the context of cancer therapy.

As previously discussed, DC based anti-cancer vaccines are currently being developed using one of two approaches: 1) the direct targeting of antigens to DCs *in vivo*; and 2) a cell-based approach that utilizes the *ex vivo* generation and antigen loading of DCs [129]. A large number of clinical studies utilize the second method, as the efficiency and control provided by *ex vivo* manipulation allows for optimal DC activation [457]. Additionally, the numbers of mature DCs in cancer patients are often decreased [46]. However, there are inherent caveats in this approach as the *ex vivo* manipulation of human cells for re-administration is highly complex and very costly [457]. In all the studies presented herein we utilized the cell-based approach to evaluate the ability of our inhibitor to enhance anti-tumor immunity. By doing so, we were able to generate mature and functional DCs while avoiding any of the potentially adverse effects that may occur as a result of PTP inhibition in other cell types. Our findings however do not rule out the possibility of administering PTP inhibitors systemically. On the contrary, we propose to actively pursue this approach in parallel. As previously discussed, the oral

administration of a PTP inhibitor was successful in delaying the onset of mammary tumorigenesis in an ErbB2 mouse model of breast cancer [267]. Importantly, the future development of this technology should not be restricted to the lymphoma and pancreatic cancer settings we tested, as DCs are capable of recognizing a wide variety of tumor antigens and are being pursued in a number of different malignancies.

In summary, the results presented here explore the roles of PTP1B and TC-PTP in DCs and detail the pre-clinical evaluation of PTP inhibitors for use in DC based cancer vaccines. Our findings represent a novel approach to boost the immune response against cancer and prompt further investigations into the clinical feasibility of this therapy.

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THE APPENDICES

APPENDIX A: Heterozgyous deletion of PTP1B protects mice from spontaneous tumor development in the Eμ-myc backround

APPENDIX B: Microarray data

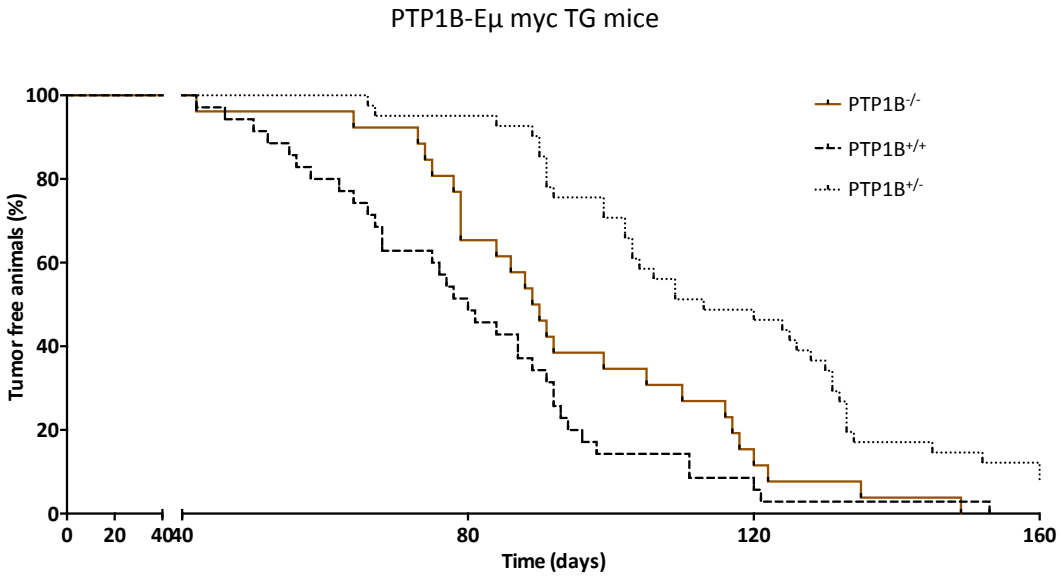

Figure A.1 Heterozgyous deletion of PTP1B protects mice from spontaneous tumor development in the Eμ-myc backround. PTP1B$^{-/-}$ mice were crossed with mice expressing the Eμ-myc transgene. Litters were backcrossed for eight generations into C57BL/6n backgrounds to generate mice with the indicted PTP1B genotypes harboring the Eμ-myc transgene. Mice were monitored for tumor formation and progression via palpation of the inguinal and axillary lymph nodes twice weekly. Kaplan-Meier analysis of survival of Eμ-myc mice carrying either WT *Ptpn1* alleles (n=34), heterozygous (n=40) or homozygous (n=26) for *ptpn1* deletion (PTP1B$^{+/-}$ vs PTP1B$^{+/+}$ ***p<0.0001).

Figure B.1 Differentially expressed genes with log fold difference ± 1.5

Of ~26,000 genes probed, 164 were found to be statistically significant after filtering for a p-value of 0.05, a T statistic of ± 2.5 and a log-fold change of ± 1.5. Values for means are presented as signal strength.

Gene symbol	Control	Inhibitor	Fold change	P-value
DDX3Y	37.55	502.00	3.74	4.68E-06
EIF2S3Y	13.07	133.95	3.36	4.03E-08
LOC100041004	27.68	146.29	2.40	1.35E-06
SLC40A1	127.99	605.51	2.24	2.63E-07
GSTM2	166.20	779.72	2.23	7.09E-09
4631433D01RIK	25.62	117.75	2.20	2.09E-09
CD72	298.36	1341.51	2.16	4.99E-07
SPIC	75.43	336.10	2.15	1.41E-07
RHOU	31.09	136.70	2.14	2.50E-05
FABP3	45.62	191.24	2.07	1.21E-07
A130065C13RIK	55.37	227.32	2.04	6.31E-08
F9	34.05	138.15	2.02	1.45E-06
PRKCB	68.84	276.87	2.01	3.02E-09
OSBPL3	202.06	790.01	1.97	1.90E-06
COL5A1	54.18	209.69	1.95	2.08E-08
NEURL2	31.28	118.17	1.92	1.64E-07
KRT17	22.87	85.33	1.92	3.00E-07
A130047F11RIK	38.00	142.21	1.91	3.52E-08
PHXR4	58.35	217.71	1.91	4.34E-08
TSPAN32	60.46	226.17	1.90	1.50E-09
B230343A10RIK	174.86	650.19	1.90	7.08E-09
CDH26	13.96	51.94	1.90	1.23E-05
GDF15	214.68	808.97	1.88	2.48E-04
JARID1D	8.68	31.92	1.87	4.11E-05
D430050H21RIK	17.47	63.73	1.87	2.45E-05
LOC100046120	141.23	513.02	1.86	3.03E-07
SLC6A9	46.31	167.81	1.85	1.68E-05
LOC331595	54.83	196.69	1.85	5.83E-07
CD81	924.00	3322.12	1.85	1.05E-09
9930031P18RIK	94.26	330.74	1.82	1.85E-07
SUSD2	35.87	127.21	1.81	9.32E-05
MSCP	84.06	291.38	1.81	1.91E-08
SCL0002975.1_346	101.97	353.80	1.79	2.62E-07
ENO2	142.45	487.19	1.79	9.27E-09

D230048P18RIK	151.59	521.86	1.79	1.01E-08
GSTM1	205.56	706.62	1.78	5.75E-08
SLC25A37	81.92	277.62	1.77	7.77E-08
F630047D10RIK	33.39	111.58	1.76	1.78E-07
GP38	239.08	796.68	1.75	1.17E-08
LIP1	129.93	428.74	1.73	3.17E-08
TRPS1	127.88	423.00	1.73	3.15E-07
TMEM140	25.79	85.18	1.72	9.61E-06
PPAP2B	106.37	349.55	1.72	2.08E-06
VAT1	162.55	530.05	1.71	4.72E-08
GSTA1	16.04	51.86	1.69	4.27E-06
ANKRD24	32.39	104.44	1.69	4.23E-08
AI646023	16.97	54.71	1.69	7.91E-05
NOS2	340.66	1092.46	1.68	3.15E-07
6820437F20RIK	159.93	505.88	1.67	9.10E-08
STAC2	19.47	60.80	1.66	1.52E-05
1110012D08RIK	177.84	560.87	1.66	1.11E-07
NPY	27.64	88.90	1.65	1.99E-03
DNMT3L	108.73	338.30	1.64	1.35E-07
B230112P13RIK	379.70	1182.28	1.64	3.29E-05
B230363H02RIK	39.54	122.37	1.63	5.76E-06
9930022F21RIK	50.82	157.34	1.63	3.11E-06
AMPD3	112.33	343.08	1.62	6.63E-09
TICAM2	146.19	448.23	1.61	5.71E-05
GNG4	33.78	102.28	1.60	5.75E-06
PLA2G4F	87.99	263.22	1.59	8.85E-09
TLR1	42.81	127.84	1.59	2.37E-06
PLA2G7	46.30	139.01	1.59	1.41E-06
LOX	130.61	408.22	1.58	6.31E-03
DTX4	48.40	144.43	1.58	6.91E-09
7530408C15RIK	68.76	203.90	1.58	2.96E-06
KIT	67.44	200.82	1.57	2.73E-06
A930023F12RIK	25.69	75.76	1.57	1.73E-07
BC030476	131.54	389.54	1.56	1.89E-06
SAMD11	27.53	81.19	1.56	2.19E-06
LOC100047788	23.34	65.99	1.55	7.88E-05
SLC32A1	37.90	110.52	1.55	3.96E-02
PTGIR	78.92	230.12	1.54	3.24E-05
4930588G05RIK	147.35	424.49	1.53	6.12E-07
4631423B10RIK	53.23	151.76	1.51	6.25E-07
EPS8L2	27.90	78.76	1.50	9.88E-06

TNFRSF9	708.49	237.91	-1.51	4.30E-03
MRPL13	1256.37	439.91	-1.52	3.29E-05
LY6C1	1569.48	551.34	-1.52	3.95E-04
H1FX	196.51	67.17	-1.53	1.85E-04
POPDC3	63.93	21.99	-1.54	7.10E-06
F2R	345.54	122.05	-1.55	1.01E-04
EAR3	83.41	28.34	-1.56	1.27E-05
B3GNT5	463.43	157.47	-1.56	9.09E-06
6430571L13RIK	141.49	47.85	-1.57	1.92E-07
HBB-B1	1912.45	647.49	-1.57	2.05E-07
CASP6	1084.02	365.48	-1.57	1.52E-08
METTL11A	288.86	96.86	-1.57	3.91E-06
2810046M22RIK	277.31	92.36	-1.58	9.61E-06
MYCL1	230.81	77.14	-1.58	1.63E-04
AI480653	120.27	41.42	-1.59	1.08E-03
SYNGR2	2812.98	930.12	-1.60	5.12E-08
SHROOM3	110.23	37.17	-1.60	4.62E-03
HBB-Y	47.48	15.67	-1.60	3.13E-06
LOC331102	462.76	151.25	-1.61	1.02E-06
EID2	148.08	48.29	-1.61	2.07E-06
FAIM3	167.00	54.50	-1.61	2.27E-06
PTMS	594.89	193.75	-1.62	1.18E-07
ACAT2	424.59	138.56	-1.62	3.93E-08
P2RY1	215.81	70.05	-1.63	1.80E-04
PTPRO	179.92	58.06	-1.63	1.42E-05
CLDN1	119.85	38.47	-1.64	1.29E-04
AMICA1	535.56	172.48	-1.64	2.33E-08
LMO2	2478.45	785.43	-1.66	1.66E-07
SERPINB2	405.35	128.86	-1.67	1.80E-04
FH1	593.83	186.09	-1.67	2.71E-05
PLA2G15	1669.13	521.19	-1.68	1.09E-05
SELL	75.01	23.26	-1.69	1.47E-06
HFE	362.35	111.29	-1.70	1.48E-07
CD69	1894.79	573.95	-1.72	8.04E-10
MFGE8	1043.93	315.96	-1.72	1.72E-07
ICOS	170.74	51.10	-1.74	4.62E-07
KCTD14	321.51	95.95	-1.74	2.33E-07
LOC233529	947.01	280.36	-1.76	1.91E-08
A930004K21RIK	70.42	20.81	-1.78	1.97E-05
PLAC8	575.24	172.85	-1.79	5.01E-06
APOL7C	1156.61	343.32	-1.80	3.88E-06

OLFM1	494.59	142.34	-1.80	1.25E-08
NGP	78.61	23.29	-1.80	6.69E-04
PTK6	63.07	18.24	-1.81	1.05E-04
SCD1	293.94	82.97	-1.82	5.02E-05
EFNA5	124.56	35.29	-1.82	4.96E-06
EBI2	511.24	143.50	-1.83	4.93E-07
SPHK1	200.31	55.68	-1.84	8.98E-07
CD79B	103.54	28.75	-1.85	1.71E-09
NEURL	134.13	37.07	-1.86	1.92E-08
PLEKHF1	285.66	78.85	-1.86	1.07E-03
HS3ST3B1	131.70	35.97	-1.87	1.06E-06
PLEK2	398.79	109.60	-1.87	1.05E-08
ALDH1A2	1348.47	362.50	-1.90	3.31E-07
2010005H15RIK	487.40	130.24	-1.91	7.95E-08
EAR6	66.47	17.66	-1.91	1.90E-04
KIF4	58.64	15.66	-1.92	3.15E-09
HHEX	317.51	84.27	-1.92	6.78E-08
EG433016	1325.05	331.04	-2.00	1.09E-06
LOC669660	168.28	42.02	-2.00	6.91E-05
IFI205	232.10	58.55	-2.01	1.29E-08
APOC2	180.27	44.39	-2.02	2.59E-07
FLT1	173.69	42.65	-2.02	1.26E-06
INSM1	267.01	65.74	-2.05	8.92E-04
CITED4	493.38	118.04	-2.08	2.86E-07
TNFRSF11B	257.35	57.67	-2.15	1.03E-05
GSN	153.23	33.94	-2.17	6.70E-07
CAR2	721.78	156.57	-2.21	5.89E-09
LY6A	1204.77	252.53	-2.24	1.38E-05
LOC666661	656.00	146.89	-2.25	8.67E-07
CYSLTR1	193.66	40.75	-2.26	2.82E-07
A430084P05RIK	859.80	178.88	-2.27	4.77E-08
SERPINA3F	306.07	66.89	-2.28	3.14E-04
MFSD2	306.00	62.64	-2.29	7.75E-05
LOC100048461	493.78	99.34	-2.33	1.54E-08
CLECSF12	526.68	103.62	-2.36	1.28E-07
MYRIP	120.47	23.23	-2.38	2.38E-09
THY1	1203.58	216.92	-2.49	1.73E-06
KMO	53.97	9.64	-2.52	3.94E-06
IFNG	194.41	33.16	-2.55	7.99E-06
LOC270152	1845.72	308.55	-2.58	2.25E-09
XIST	81.93	13.25	-2.63	4.86E-06

FAM115C	2442.41	343.59	-2.83	4.13E-11
IL12RB2	278.22	38.69	-2.88	2.03E-07
LOC100042514	202.74	16.83	-3.02	3.88E-02
CLDN11	154.05	17.43	-3.14	1.05E-11
EN2	320.55	36.04	-3.17	3.07E-08
MGL2	683.37	60.81	-3.49	1.85E-08
ARG1	238.28	19.33	-3.61	1.34E-06