Protein tyrosine phosphatases regulate cancer bioenergetics and macrophage immune responses

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LIST OF ABBREVIATIONS

AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BMDM	bone marrow-derived macrophages
CAC	colitis-associated colorectal cancer
CAC	citric acid cycle
CD	Crohn's disease
CDC	cell division cycle
CDK	cyclin-dependent kinase
CPM	counts per million
CRC	colorectal cancer
CSF1R	colony stimulating factor 1 receptor
DC	dendritic cell
DNA	deoxyribonucleic acid
DSP	dual-specificity phosphatase
DSS	dextran sodium sulfate
ECAR	extracellular acidification rate
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ETC	electron transport chain
EYA	eyes absent homolog
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FMO	fluorescence minus one
GBP	guanylate-binding protein
GC-MS	gas chromatography mass spectrometry
GF	growth factor

GO	gene ontology
HET	heterozygous
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IFI	interferon-inducible
IFN	interferon
IFNGR	interferon gamma receptor
IGF1R	insulin-like growth factor 1 receptor
IL	interleukin
IMM	inner mitochondrial membrane
INSR	insulin receptor
IRF	interferon regulatory factor
JAK	janus kinase
KO	knockout
LMW-PTP	low molecular weight PTP
LPS	lipopolysaccharide
M-CSF	macrophage colony stimulating factor
mRIPA	modified radioimmunoprecipitation assay
MAPK	mitogen-activated protein kinase
MFI	mean fluorescence intensity
MKP	MAPK phosphatase
MTMR	myotubularin-related proteins
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
NO	nitric oxide
OCR	oxygen consumption rate
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

PDGFR	platelet-derived growth factor receptor
PMN	polymorphonuclear cell
PPP	pentose phosphate pathway
PRL	phosphatase of regenerating liver
PTEN	phosphatase and tensin homolog
PTM	post-translational modification
PTP	protein tyrosine phosphatase
PTP1B	protein tyrosine phosphatase non receptor type 1
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
RNA-Seq	RNA-sequencing
ROS	reactive oxygen species
RT	room temperature
RTK	receptor tyrosine kinase
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SITA	stable isotope tracer analysis
SNP	single nucleotide polymorphism
SOCS	suppressor of cytokine signaling
SRC	spare respiratory capacity
SSH	slingshot protein phosphatases
STAT	signal transducer and activator of transcription
TAM	tumour-associated macrophage
TC-PTP	T cell protein tyrosine phosphatase
TCA	tricarboxylic acid cycle
TCL	total cell lysate
UC	ulcerative colitis
VEGFR2	vascular endothelial growth factor receptor 2 (also known as KDR)
WT	wild-type
WTCCC	Wellcome Trust Case Control Consortium

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ENGLISH ABSTRACT

Colorectal cancer (CRC) is the third deadliest cancer worldwide and there is still much to uncover about the underlying mechanisms for its initiation and progression. We now understand that certain acquired cellular characteristics are commonly present in a plethora of different cancers. Two of these hallmarks of cancer that are closely intertwined are dysregulated cellular metabolism and inflammation. Considering that protein tyrosine phosphatases (PTPs) can act as both oncogenes or tumour suppressors, we sought to investigate their role in regulating these two cancer hallmarks.

In Chapter 2, I investigated the role of PTPs in regulating the cellular metabolism of colorectal cancer cells and identified 24 PTP candidates. Additional studies validated that the candidate TC-PTP is indeed a positive regulator of mitochondrial respiration and ATP production in CRC cells. Absence of TC-PTP does not affect the glycolytic activity of cells, but leads to hyperphosphorylation of STAT1, STAT3 and p38.

In Chapter 3, I demonstrated that TC-PTP is an important modulator of macrophage polarization and immune response. Inhibition of TC-PTP in macrophages promotes pro-inflammatory macrophage polarization and leads to a dysregulated transcriptome, with over-representation of immune response and mitochondrial respiration pathways.

In Chapter 4, we established that PTP1B is a regulator of the IL-10-induced transcriptome in macrophages. Deficiency of PTP1B in macrophages activates STAT3 and leads to induction of anti-inflammatory genes. We also identified a subset of STAT1-induced pro-inflammatory genes that are increased in absence of PTP1B, highlighting its importance in maintaining the homeostasis between pro- and anti-inflammatory cytokine signaling.

The work presented in this thesis identifies a role for PTPs as negative regulators of cellular metabolism and tumour-promoting inflammation. TC-PTP may act as a tumour suppressor by preventing the metabolic switch known as the Warburg effect and by promoting anti-inflammatory macrophage polarization. Given that altered metabolism can induce inflammation and vice-versa, further studies would need to be conducted to understand how cellular metabolism and macrophage immune response are interconnected in the context of colorectal cancer and colitis-associated colorectal cancer.

RÉSUMÉ

Le cancer colorectal (CCR) est le troisième cancer le plus mortel au monde et les mécanismes qui favorisent son initiation et sa progression demeurent peu connus. Nous savons maintenant que certaines caractéristiques cellulaires acquises sont présentes dans plusieurs types de cancer, incluant deux qui sont intrinsèquement liées: le dérèglement du métabolisme cellulaire ainsi que l'inflammation pro-tumorale. Puisque les protéines tyrosine phosphatases (PTPs) peuvent agir comme oncogènes et suppresseurs de tumeur, nous avons cherché à investiguer leur rôle dans la modulation de ces deux critères dans les cellules cancéreuses.

Dans le Chapitre 2, j'ai ciblé l'ensemble des PTPs au niveau des cellules du CCR et identifié 24 candidats qui affecte leur métabolisme. Des études supplémentaires ont confirmé que TC-PTP régule de façon positive la respiration mitochondriale ainsi que la production d'ATP dans ces cellules. L'absence de TC-PTP n'affecte pas la glycolyse, mais mène à une hyperphosphorylation de STAT1, STAT3 et p38.

Dans le Chapitre 3, j'ai démontré que TC-PTP est un modulateur important de la polarisation et la réponse immunitaire des macrophages. L'inhibition de TC-PTP dans les macrophages favorise leur polarisation pro-inflammatoire et mène à une dérégulation de leur transcriptome, principalement des voies impliquant la réponse immunitaire, la fonction des macrophages et la respiration mitochondriale.

Dans le Chapitre 4, nous avons établi que PTP1B est un régulateur du transcriptome des macrophages induit par IL-10. L'inhibition de PTP1B active STAT3 et résulte à l'induction de gènes anti-inflammatoires. Nous avons également identifié un ensemble de gènes pro-inflammatoires induits par STAT1 en l'absence de PTP1B, soulignant son importance à maintenir l'homéostasie des gènes pro- et anti-inflammatoires.

Les travaux présentés dans cette thèse identifient un rôle pour les PTPs comme régulateurs négatifs du métabolisme et de l'inflammation pro-tumorale. TC-PTP pourrait être un suppresseur de tumeur en prévenant le changement métabolique, nommée l'effet Warburg, et en favorisant la polarisation anti-inflammatoire des macrophages. Puisqu'une dérégulation du métabolisme peut induire l'inflammation et vice-versa, d'autres études devraient être effectuées pour comprendre comment le métabolisme et la réponse immunitaire des macrophages sont connectés dans le contexte du CCR.

ACKNOWLEDGEMENTS

The first person that I would like to thank is my supervisor Michel. Thank you for accepting me in your lab, even though I did not realize that we were having an interview, and for trusting me and giving me the opportunity to begin a new project that I really wanted to do. Your kindness is unparalleled in this dog-eat-dog world. I must also thank Kelly deeply who, by giving me the opportunity to work with her on the macrophage project, has thought me so much and showed me what a superhero mom scientist looks like. I have never met such a fierce and passionate scientist.

My whole PhD degree would have been much more difficult without the help of precious people in the lab, and I must thank them all. Jean-François, for being the best lab manager and always answering all my questions. Serge, for your unending support and wealth of knowledge by which I am always flabbergasted. You seem to know the answer to all questions, regardless of the topic. Isabelle, for general support, for your extensive knowledge about PCR, for laughter and for being such a fun person and a nice bench neighbour. Jacinthe for general help and good spirit. Noriko for being the mouse whisperer, official figure-making guru and for your willingness to help. A lab without a core would not be a lab at all. A special thanks to Rosalie and Audrée from the mouse facility who have relieved me from much animal surveillance and have been very helpful.

The PhD experience would also be completely different if the lab members were not so special. Not only colleagues, but most of all friends. Teri, Elie, Eugene, Serge, Tzvet, João and all the undergrads that have passed through the lab. A special thanks to Hayley who has worked with me for over a year and been very helpful. To Emily and Chadi who have left but are never gone, I am glad that I met you. Your friendship is something rare. To all the friends that I've made throughout the years in the centre, you've made it worth it when experiments were not working out! All those discussions over beers are priceless.

I must also thank my parents, Claude and Johanne, and sisters, Karine and Maude, for their never-ending support and encouragement along this long road. Understanding when I couldn't make it home, sending me love. They make me prouder than they know.

And last but not least, my love. How I am glad that I came back to McGill to do my PhD. You have been my main source of moral support, my rock, my number one fan. I cannot thank you enough for all that you have done for me. Thank you.

PREFACE AND CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

This thesis was written in the manuscript format. Data will be presented in three separate result chapters (Chapter 2, Chapter 3 and Chapter 4).

CHAPTER 2 – A HUMAN PHOSPHATOME METABOLIC SCREEN IDENTIFIES TC-PTP AS A REGULATOR OF COLORECTAL CANCER BIOENERGETICS

Vinette, V.*, Insull, H., Sacher, E. and Tremblay, M.L. (2018).* TC-PTP emerges as a regulator of colorectal cancer bioenergetics through a human phosphatome screen*. Manuscript to be submitted* September 2018.

Preface: This chapter consists in work that I performed following the obtention of an oncometabolism grant to investigate the role of PTPs in modulating the Warburg effect. Experiments are almost completed, and we will submit this paper in September 2018.

Contribution: I designed the experiments, performed all metabolic assays (Seahorse screen, ATP assays, proliferation assays), did qRT-PCR and western blot experiments and analyzed the data. I wrote, edited and reviewed the manuscript. HI performed western blots and qRT-PCR experiments, as well as analyzed some data. ES helped generate the stable cell lines and performed qRT-PCR experiments. MLT participated in reading and editing the manuscript. HI performed figure 2.6. I performed figures 2.1, 2.2, 2.3, 2.4, 2.5, 2.7 and 2.8.

Original contributions to knowledge: In this paper, I established the following:

1. 24 members of the PTP family regulate the metabolism of colorectal cancer cells.
2. Inhibition of TC-PTP leads to downregulation of mitochondrial respiration and ATP production, with no change in glycolysis.
3. STAT1, STAT3 and p38 are hyperphosphorylated in TC-PTP-deficient colorectal cancer cells.
4. This study proposes that PTPs can act as negative or positive regulators of the Warburg effect by modulating cellular mitochondrial respiration.

CHAPTER 3 – TC-PTP REGULATES THE POLARIZATION AND IMMUNE RESPONSE OF MACROPHAGES

***Vinette, V.**, Zolotarov, Y., Insull, H. and Tremblay, M.L. (2018).* TC-PTP regulates the polarization and pro-inflammatory immune response of macrophages. *Manuscript in preparation.*

Preface: This chapter consists in work that I performed starting 4 years into my PhD. Although quite advanced, we plan to finish experiments by the beginning of the year and submit the manuscript shortly thereafter.

Contribution: I designed the experiments, performed all mouse, flow cytometry and qRT-PCR experiments, as well as generated and validated the LysM-Cre mouse model. I prepared RNA samples for RNA-Seq, performed downstream data analysis for RNA-Seq and wrote and reviewed the manuscript. YZ did the bioinformatics analysis of the raw RNA-Seq data, generated heatmaps and participated in the writing and editing of the manuscript. HI performed some western blots with LysM-Cre mouse samples. MLT participated in reading and editing the manuscript. YZ analyzed the raw data from RNA-Seq that led to figures 3.5 to 3.7 and tables 3.1 to 3.4. HI did figure 3.9. I performed figures 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8 and 3.10, as well as tables 3.1, 3.2, 3.3 and 3.4.

Original contributions to knowledge: In this paper, I made the following discoveries:

1. Deficiency of TC-PTP promotes macrophage polarization towards the pro-inflammatory M1 phenotype.
2. TC-PTP loss in M1 macrophages leads to a dysregulated transcriptome, with an over-representation of pathways involved in immune response, macrophage function and metabolism, particularly mitochondrial respiration.
3. Myeloid-specific deletion of TC-PTP is sufficient to promote M1 macrophage polarization.

CHAPTER 4 – PTP1B IS A REGULATOR OF THE INTERLEUKIN 10-INDUCED TRANSCRIPTIONAL PROGRAM IN MACROPHAGES

Pike, K.A., Hutchins, A.P.*,* ***Vinette, V.****, Théberge, J.F., Sabbagh, L., Tremblay, M.L. and Miranda-Saavedra, D. (2014).* Protein tyrosine phosphatase 1B is a regulator of the IL-10- induced transcriptional program in macrophages*. Science Signaling* ***7****(324): ra43.*

Preface: This chapter was published in *Science Signaling* in 2014. I worked closely with the co-first author Dr. Pike and we performed many mouse experiments together.

Contribution: I performed most mouse experiments with KAP and extracted RNA from macrophages to perform all quantitative real-time PCR (qRT-PCR) studies. I also designed the mouse primers to perform qRT-PCR, analyzed the data and participated in writing and reviewing the manuscript. KAP designed all the experiments, performed all mouse experiments, analyzed the data, and wrote and reviewed the manuscript. APH performed bioinformatics analyses of the RNA-sequencing data and participated in reading and revising the manuscript. LS participated in some mouse experiments and data analysis, as well as reviewing the manuscript. JFT performed RNA extraction for the samples to be sent for RNA-sequencing. MLT and DMR participated in reading and editing the manuscript. I performed figures 4.2C, 4.2D, 4.3A, 4.4B and 4.6C.

Original contributions to knowledge: In this paper, we made the following discoveries:

1. PTP1B negatively regulates the IL-10/STAT3 pathway and not the IL-6/STAT3 pathway.
2. Inhibition of PTP1B in macrophages leads to hyperactivation of anti-inflammatory genes upon stimulation with IL-10.
3. Loss of PTP1B in macrophages leads to upregulation of a subset of pro-inflammatory interferon-regulated genes as a result of STAT1 hyperphosphorylation.
4. PTP1B regulates the IL-10-induced transcriptome in macrophages.

ADDITIONAL PUBLICATIONS

Pearl, D., Amiri, M., **Vinette, V.**, Beug, S., Kim, S.H., Jones, L., Robichaud, N., Jia, J.J., Ali, H., Tremblay, M.L., Alain, T., Morita, M., Tahmasebi, S. and Sonenberg, N. (2018). 4E-BP-dependent Translational Control of Macrophage Polarization and Inflammatory Response. Submitted to *Journal of Experimental Medicine.*

Coulis, G., Shi, Y., Labbé, D.P., Bergeron, A., **Vinette, V.**, Karsenty, G., Tremblay, M.L., Tardif, J.C. and Boivin, B. (2018). PTP1B is a Regulator of Gene Silencing and Cardiac Hypertrophy in Mice. To be submitted Fall 2018.

Penafuerte, C. *, Perez-Quintero, L.A. *, **Vinette, V. *,** Hatzihristidis, T., Tremblay, M.L. (2017) Mining the Complex Family of Protein Tyrosine Phosphatases for Checkpoint Regulators in Immunity. *Curr Top Microbiol Immunol* **4**(10): 191-214.

* These authors contributed equally to the work.

Bussières-Marmen, S., **Vinette, V.**, Aubry, I. and Tremblay, M.L. (2017). The protein tyrosine phosphatase TC-PTP helps maintain the gut epithelium barrier through its function in stem cell proliferation. *Cell Mol Immunol* **13**: 1-10.

Penafuerte, C., Feldhammer, M., Mills, J., **Vinette, V.**, Pietrobon, A., Desai, N., Zogopoulos, G. and Tremblay, M.L. (2017). Downregulation of PTP1B and TC-PTP phosphatases potentiate dendritic cell-based immunotherapy through IL-12/IFN gamma signalling. *Oncoimmunology* **6**(6): e1321185.

Labbé, D.P., Uetani, N., **Vinette, V.**, Lessard, L., Aubry, I., Mignon, E., Sirois, J., Haigh, J., Bégin, L.R., Trotman, L.C., Paquet, M. and Tremblay, M.L. (2016). PTP1B deficiency enables the ability of a high fat diet to drive the invasive character of PTEN-deficient prostate cancers. *Cancer Research* 76(11): 3130-3135.

INTRODUCTION

Rationale

Colorectal cancer is the third deadliest cancer in the world and it is exacerbated by chronic inflammation. Characteristic drivers of tumour cells have been established, with dysregulated cancer bioenergetics and tumour-promoting inflammation being emerging hallmarks of cancer that favour its initiation or progression. Considering that metabolic and immune pathways can induce each other, it is important to understand what regulates these processes. TC-PTP and PTP1B are two known protein tyrosine phosphatases that are involved in the regulation of inflammation, colitis, metabolism and cancer. Hence, the work presented in this thesis is based on the hypothesis that *metabolism and inflammation are processes that are intimately linked, and that protein tyrosine phosphatases can regulate both to modulate cancer progression*. As such, studying the involvement of protein tyrosine phosphatases in metabolic and immune pathways may provide novel therapeutic strategies based on metabolic and immune regulation of cancer immunotherapy.

Objectives

To study the above-mentioned hypothesis, my doctoral project is divided into the following objectives:

1. Determine if any member of the protein tyrosine phosphatase superfamily modulates cellular metabolism of colorectal cancer cells by regulating the Warburg effect.
2. Investigate the role of TC-PTP in regulating the polarization and immune response of macrophages.
3. Examine whether deletion of TC-PTP specifically in myeloid cells is sufficient to drive inflammation and disease.
4. Investigate the role of PTP1B in controlling the immune response and cytokine signaling of macrophages.

CHAPTER 1

LITERATURE REVIEW

1.1 Protein Tyrosine Phosphatases

Phosphorylation and dephosphorylation events are some of the most common post-translational modifications (PTMs) in cells, and these are catalyzed by protein kinases and protein phosphatases, respectively. One of the major group of enzymes responsible for dephosphorylation is the protein tyrosine phosphatase (PTP) family, initially discovered by Nick Tonks and colleagues (1). This superfamily consists of 106 members that mostly remove phosphate groups from tyrosine residues of their specific substrates, while other members can remove phosphatase groups from serine and threonine residues. This family is divided into four classes based on the amino acid sequence of their catalytic domain (2-4). The defining feature of PTPs is that they share a common HCX_5R catalytic motif, known as the *PTP signature motif*. Residues in this PTP motif form the phosphatase binding loop, or P-loop, located at the bottom of the active site (5, 6). In this HCX_5R motif is a cysteine (Cys, C) residue that is located at the bottom of the catalytic cleft of PTPs. Another important residue is the aspartate (Asp, D) residue which is part of a highly conserved WPD motif located on the side of the catalytic cleft and is essential for the two-step mechanism of action of PTPs (**Figure 1.1**).

1.1.1 Mechanism of action

The first step is initiated by a nucleophilic attack by the cysteine of the active site on the phosphorus atom of the bound substrate. At the same time, a well-positioned general acid residue, commonly Asp181, donates its proton to the leaving group oxygen (6). The phosphate group remains covalently attached to the nucleophile in this first substitution reaction (7, 8). In the second step of the mechanism, the WPD loop closes over the phosphorylated substrate and allows for the removal of the phosphate from the

phosphoenzyme intermediate, with a water molecule acting as the nucleophile. It is the specific aspartate residue within the loop that enables the proper recognition of the substrate (9, 10). Mutations that affect the WPD loop prevent the closing of the loop over the substrate in the active pocket, leading to inefficient reaction and dephosphorylation of the substrate (10, 11). It is the dept of the catalytic cleft that dictates the specificity towards substrates containing tyrosine-phosphorylated residues as opposed to the shorter serine- and threonine-phosphorylated residues (4-6, 12).

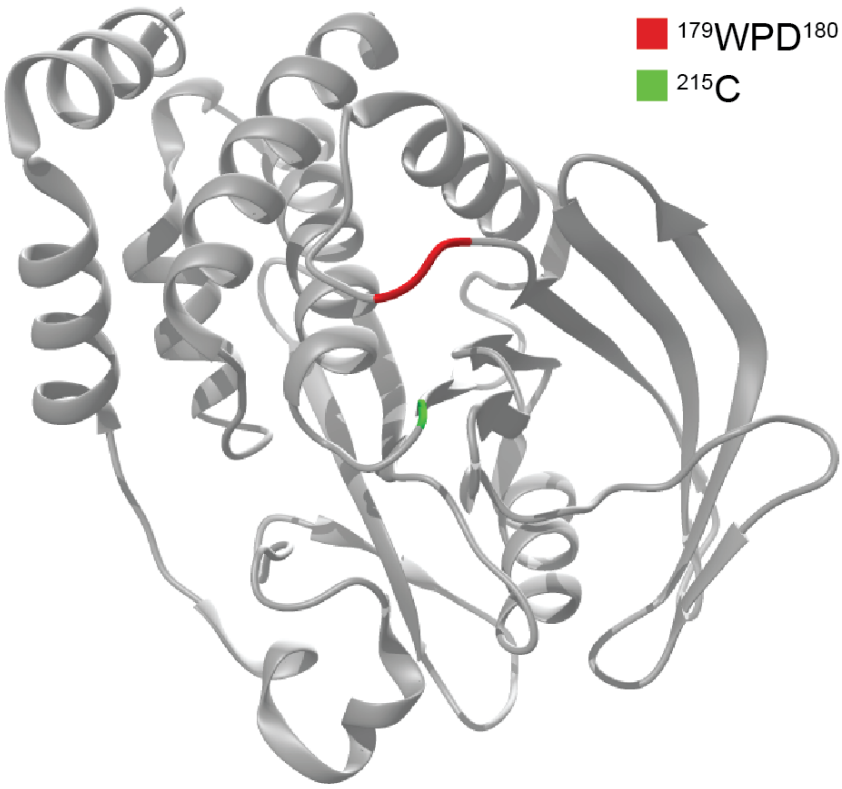

Figure 1.1: Structure of the original protein tyrosine phosphatase PTP1B. For PTP1B, the nucleophilic attack is done by the cysteine from the P-loop in the active site and is the residue 215 (^{215}C, green). The WPD loop essential for the removal of the phosphate group is located within residues 179-180 (^{179}WPD^{180}, red). Figure generated by Noriko Uetani using protein database ID (PDB ID, structure 3A5J) and UCSF Chimera 1.10.2 (13, 14).

There are two predominant mutations that can abolish the catalytic activity of PTPs: mutating the critical cysteine in the PTP signature motif to serine (C to S mutant, or C/S) or mutating the aspartate residue of the WPD loop to alanine (D to A mutant, or D/A). These mutants can be used in substrate-trapping experiments, which is a well-established and commonly used tool to identify novel PTP substrates (15, 16). There are three main criteria for a protein to be considered a substrate of a PTP: 1) The tyrosine phosphorylation of the substrate *must* be modulated by the PTP, 2) The PTP *must* dephosphorylate the candidate substrate *in vitro*, and 3) The C/S or D/A substrate-trapping mutants must efficiently capture the candidate substrate *in vitro* and this must be a direct interaction (15, 16).

1.1.2 Different classes of PTPs

PTPs are divided into four classes based on the amino sequence of their catalytic domain (**Figure 1.2**). Class I consists of 98 members divided into the classical PTPs (37 members) and the dual-specificity phosphatases (DSPs, 61 members). The classical PTP are divided into subclasses: the receptor PTPs and the non-receptor PTPs. The DSPs are further subdivided into seven groups: atypical DSPs, MAPK phosphatases (MKPs), phosphatases of regenerating liver (PRLs), CDC14s, myotubularin-related proteins (MTMRs), slingshot protein phosphatases (SSHs) and phosphatase and tensin homologs (PTENs). Class II contains one low molecular weight PTP (LMW-PTP). Class III comprises the CDC25s and Class IV is composed of the eyes absent homologs (EYAs) members (2, 3). Many of the most commonly-studied PTPs, such as PTP1B, TC-PTP, PTP-PEST and PTPRS are part of the classical PTPs in Class I.

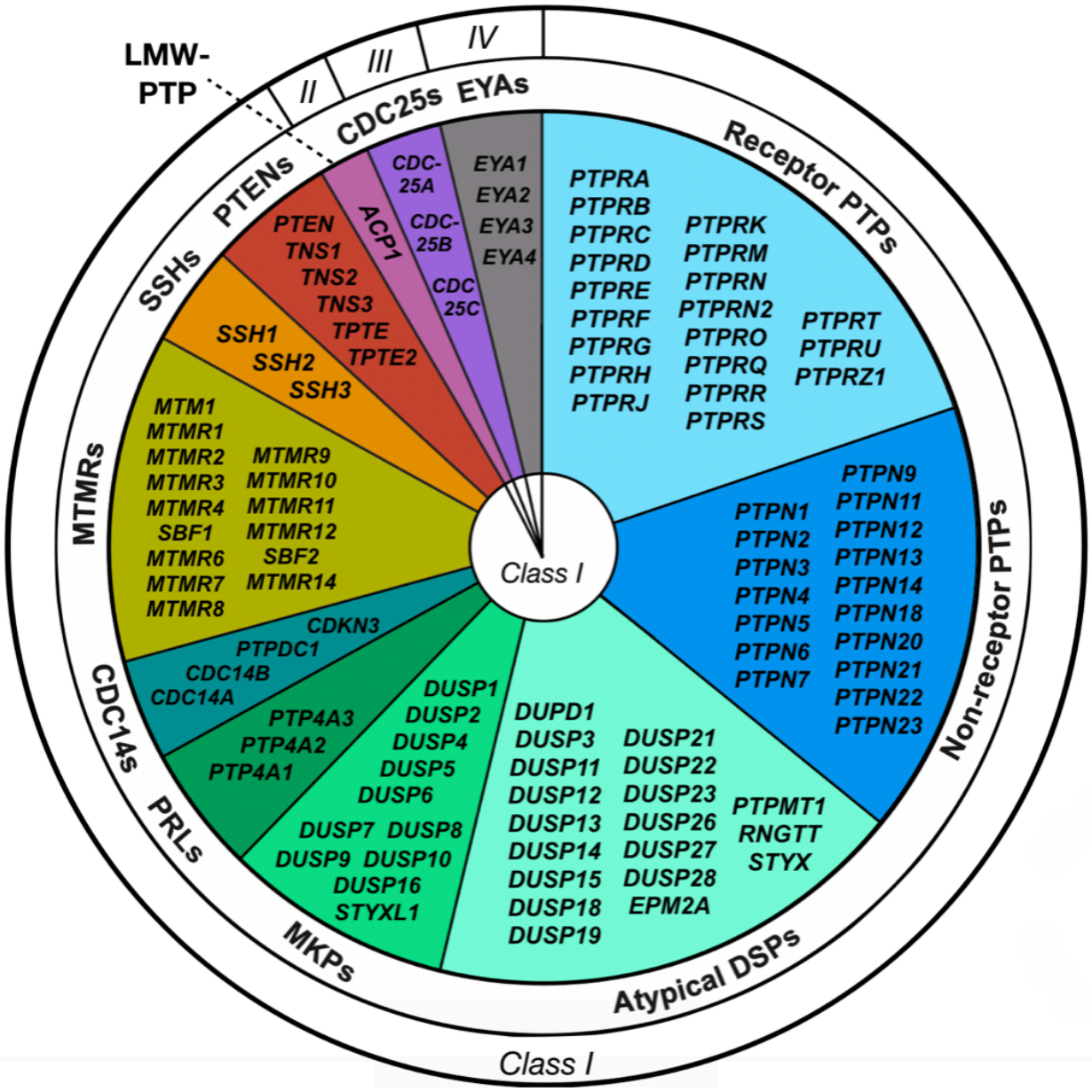

Figure 1.2: Family of protein tyrosine phosphatases. According to the newest nomenclature and classification, there are currently 106 PTP members. Class I comprises 37 classical PTPs divided into 20 receptor and 17 non-receptor PTPs, as well as 61 dual-specificity phosphatases (DSPs), which are further divided into 19 atypical DSPs, 11 MKPs (MAPK phosphatases), 3 phosphatases of regenerating liver (PRLs), 4 CDC14s, 15 myotubularin-related proteins (MTMRs), 3 slingshot protein phosphatases (SSHs) and 6 phosphatase and tensin homologs (PTENs). Class II includes one member, a low-molecular weight PTP (LMW-PTP). Class III includes the 3 CDC25s and Class IV contains the 4 eyes absent homolog (EYA) members.

1.1.2.1 Class I: Classical PTPs and DSPs

The first class of PTPs constitute the founding group and are Cys-based tyrosine phosphatases, meaning that the nucleophilic attack is performed by a cysteine residue (3, 17, 18). Most members of this family have a conserved amino acid sequence and protein folding of their catalytic PTP domain. They possess the highly conserved HCX_5R signature motif present in most PTPs (2, 3).

The classical PTPs belonging to the first class are subdivided into two subgroups: receptors PTPs and non-receptor PTPs. As their name implies, receptors PTPs are located at the cell surface or in some cases at the membrane of different organelles. This subclass includes 20 members: *PTPRA, PTPRB, PTPRC, PTPRD, PTPRE, PTPRF, PTPRG, PTPRH, PTPRJ, PTPRK, PTPRM, PTPRN, PTPRN2, PTPRO, PTPRQ, PTPRR, PTPRS, PTPRT, PTPRU* and *PTPRZ1.* Non-receptor PTPs are generally located in the cytoplasm of cells, specifically in the endoplasmic reticulum (ER) or nucleus of cells. This subfamily contains 17 members: *PTPN1, PTPN2, PTPN3, PTPN4, PTPN5, PTPN6, PTPN7, PTPN9, PTPN11, PTPN12, PTPN13, PTPN14, PTPN18, PTPN20, PTPN21, PTPN22* and *PTPN23* (2, 3).

In contrast to the rest of the family members, the DSPs that belong to Class I are able to dephosphorylate not only tyrosine residues, but also serine and threonine residues, making them more versatile (2, 4, 19). They are separated into 7 subclasses: atypical DSPs, MAPK phosphatases (MKPs), phosphatases of regenerating liver (PRLs), CDC14s, myotubularin-related phosphatases (MTMRs), the slingshot homolog phosphatases (SSHs) and the phosphatase and tensin homologs (PTEN). The atypical DSPs contain 19 members: *DUPD1*, *DUSP3*, *DUSP11*, *DUSP12*, *DUSP13*, *DUSP14*,

DUSP15, *DUSP18*, *DUSP19*, DUSP21, DUSP22, DUSP23, *DUSP26*, *DUSP27*, *DUSP28*, *EPM2A*, *PTPMT1*, *RNGTT* and *STYX*. The MKPs contain the following 11 members: *DUSP1*, *DUSP2*, *DUSP4*, *DUSP5*, *DUSP6*, *DUSP7*, *DUSP8*, *DUSP9*, *DUSP10*, *DUSP16*, *STYXL1*. The PRLs consist of 3 members: *PTP4A1*, *PTP4A2* and *PTP4A3*. The CDC14s contain 4 members: *CDC14A*, *CDC14B*, *CDKN3* and *PTPDC1*. The MTMRs is a larger subclass with 15 members: *MTM1*, *MTMR1*, *MTMR2*, *MTMR3*, *MTMR4*, *SBF1*, *MTMR6*, *MTMR7*, *MTMR8*, *MTMR9*, *MTMR10*, *MTMR11*, *MTMR12*, *SBF2* and *MTMR14*. The SSHs consists of only 3 members: *SSH1*, *SSH2* and *SSH3*, while the PTENs contain 6 members: *PTEN*, *TNS1*, *TNS2*, *TNS3*, *TPTE* and *TPTE2* (2, 3).

1.1.2.2 Class II: LMW-PTP

The second class of PTPs is also Cys-based contains only one memer: *ACP1*. It is named low molecular weight PTP (LWM-PTP) due to its small size. It has an N-terminal HCX_5R signature motif common to most PTPs. ACP1 has a large open catalytic pocket that can accommodate larger tyrosine-phosphorylated substrates (3).

1.1.2.3 Class III: CDC25s

Class III of the PTP superfamily contains three members of the cell division cycle (CDC) 25 subfamily: *CDC25A*, *CDC25B* and *CDC25C*. Members from this class are structurally different from the previous two classes since their catalytic domain is a rhodanese domain (20, 21), and they also lack a PTP domain. However, they do contain the conserved HCX_5R catalytic motif (3). Interestingly, members of this class lack the WPD loop. Instead, it has been proposed that the initial nucleophilic attack involves the

monoprotonated phosphate substrate, and a conserved glutamate residue (Glu, also called glutamic acid) next to the catalytic Cys residue has been proposed to be involved in the catalysis (3, 22, 23). As their name implies, CDC25s regulate cell cycle progression and checkpoint pathways that control the cellular response to DNA damage by activating cyclin-dependent kinases (CDKs) through dephosphorylation of residues in the ATP-binding loop of CDKs (3, 24).

1.1.2.4 Class IV: EYAs

The eyes absent (EYAs) members compose the fourth class of PTPs, and these include *EYA1*, *EYA2*, *EYA3* and *EYA4*. The four EYA proteins do not contain the conserved HCX_5R catalytic motif and are not Cys-based tyrosine phosphatases, but are rather Asp-based (3). They are so-called due to a highly conserved C-terminal domain, called the EYA domain, that is involved in DNA binding (25). Members of this class have both a tyrosine (Tyr, Y) and threonine (Thr, T) phosphatase activity. For Tyr dephosphorylation, the mechanism of action of EYAs involves an Asp acting as the nucleophile as opposed to a Cys like most other PTPs, and utilizes Mg^{2+} as a cofactor (2, 26). For the Thr dephosphorylation, catalysis is performed by another active site located in the N-terminal domain (27, 28). As such, EYAs have a dual Tyr/Thr phosphatase activity based on two separate catalytic domains (3).

1.1.3 Regulation of PTPs

PTPs can be regulated by several PTMs, including sumoylation, prenylation and proteolytic cleavage, which can modulate PTP enzymatic activity (10, 29-35). It has been demonstrated that GP63, a protease present on the cell surface of *Leishmania* parasites,

is able to cleave various PTPs, notably PTP1B (gene name *PTPN1*), TC-PTP (gene name *PTPN2*), SHP-1 (gene name *PTPN6*) and PTP-PEST (gene name *PTPN12*) during infection (36-38). Several non-receptor PTPs belonging to class I have also been shown to be cleaved by proteolysis during tumour formation (39), emphasizing the importance of proteolysis in regulating the activity of PTPs in the context of disease progression and cancer. However, the two most common PTMs responsible for mediating PTPs enzymatic activity are phosphorylation and reversible oxidation.

1.1.3.1 Phosphorylation

Phosphorylation is a common regulatory checkpoint of PTPs, especially serine (Ser) phosphorylation, which has been shown to negatively or positively regulate the activity of various PTPs, depending on the context. For instance, phosphorylation on Ser39/Ser434 on PTP-PEST by protein kinase A (PKA) or protein kinase C (PKC) negatively regulates its activity (40). Similarly, serine phosphorylation of SHP-1 and PTP1B inhibits their phosphatase catalytic activity (41, 42). On the other hand, serine phosphorylation of CD45 (gene name *PTPRC*) and PTPRA results in an upregulation of their catalytic activity (43, 44). Phosphorylation of TC-PTP on Ser304 by cyclin-dependent kinases has been demonstrated during mitosis, although the effect of this phosphorylation is not quite known (45). Tyrosine phosphorylation has also been established to modulate PTP activity, although it is less frequent. As an example, injection of insulin in mice induces the tyrosine phosphorylation of PTP1B, leading to a reduction in its activity (10, 46). Hence, phosphorylation of PTPs by receptor tyrosine kinases (RTKs) is a crucial PTM that modulates their function and activity.

1.1.3.2 Reversible oxidation

In cancer cells, there is generally an increase in reactive oxygen species (ROS) levels as a consequence of the cross-talk between infiltrating immune cells and metabolic pathways. They can also be intrinsically produced by cancer cells themselves following different events, such as increased metabolic activity, mitochondrial dysfunction, peroxisome activity or oncogene activity (10, 47-49). The transient and localized modulation of ROS levels within cancer cells can modulate several hallmarks of cancer, including proliferation and apoptosis (50). The ROS hydrogen peroxide (H_2O_2) has been shown to regulate the activity of certain PTPs, notably PTPRA and PTP1B, through their reversible oxidation (51-53). These reports have established that reversible oxidation is important to control the steady-state levels of tyrosine phosphorylation in the cell at a given time (10).

1.1.4 PTP1B

Protein tyrosine phosphatase non-receptor type 1 (PTP1B) is the founding member of the PTP superfamily. It was extracted and purified in 1988 from human placenta by Nick Tonks (1, 54). PTP1B is a non-receptor PTP that is encoded by the gene *PTPN1* located on chromosome 20q13.13 in humans. It is composed of a N-terminal catalytic domain, two proline-rich motifs and a C-terminal hydrophobic region. PTP1B has a cytoplasmic domain that is anchored to the cytoplasmic face of the ER through its C-terminal hydrophobic region (55). It has many substrates, including JAK2 (56-59), TYK2 (56), STAT6 (60), CSF1R (61), EGFR (16, 62, 63), PDGFR (62-64), INSR (65-70) and IGF1R (66, 71) (**Table 1.1**).

1.1.4.1 PTP1B mouse models

Several PTP1B mouse models have been generated in the past two decades. The first was generated in 1999 by Elchebly and colleagues and was a systemic deletion of PTP1B. These mice were resistant to diet-induced obesity and had increased sensitivity to insulin (68). Given that the insulin receptor (INSR) is a substrate of PTP1B, PTP1B knockout mice had an increased and prolonged tyrosine phosphorylation of the INSR. Considering that obesity is a risk factor for type II diabetes, this PTP1B mouse model proposed a novel treatment for the treatment of type II diabetes and obesity since deletion of PTP1B ameliorated both of these phenotypes (68). We have also shown with this mouse model that PTP1B-deficient mice have an increase in the monocyte/granulocyte ratio. Furthermore, upon stimulation with macrophage colony stimulating factor (M-CSF), a growth factor essential for macrophage differentiation, bone marrow cells derived from these mice generated more monocytic colonies compared to control mice (61).

A mouse model with a neuronal deletion of PTP1B was generated several years later to try to establish the mechanism of action of PTP1B in regulating obesity. These mice did in fact display reduced weight and adiposity, as well as an increase in activity and energy expenditure (72). Interestingly, these mice were hypersensitive to leptin, a hormone secreted by adipocytes that acts on the hypothalamus in the brain to inhibit food intake and increase energy expenditure, even though leptin levels were found to be high. This model conveniently established that neuronal deletion of PTP1B regulates adipocyte leptin production that could be essential for the development of leptin resistance, thus proposing that body mass and adiposity was regulated through actions in the brain (72).

1.1.5 TC-PTP

T cell protein tyrosine phosphatase (TC-PTP) is encoded by the gene *PTPN2* located on chromosome 18p11.2 – 11.3 in humans, and in a syntenic region in mice (73). The promoter of TC-PTP does not contain a TATAA or CAAT box. Rather, transcription initiation seems to occur through the binding of transcription factors to a 5'-CA repeat and GC-rich regions. Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) and ETS binding sites also exist in the initiator region of *PTPN2* (74). TC-PTP is 70% identical to PTP1B based on the sequences of their catalytic domain, and their structures are closely related (75, 76). Although it was originally cloned from a peripheral human T cell library, TC-PTP is ubiquitously expressed throughout different tissues and cell types, but more strongly expressed in lymphoid cell lineages (77).

TC-PTP comprises two isoforms that have different subcellular localizations: a 45 kDa and a 48 kDa isoform. The most predominant isoform in the cell is the 45 kDa isoform (TC45), which is composed of 10 exons and contains a RKRKR nuclear signature that targets it to the nucleus, and preferentially to chromatin. This isoform is able to shuttle between the nucleus and the cytoplasm following various stresses and can then dephosphorylate plasma membrane receptors. For instance, in response to epidermal growth factor receptor (EGFR) accumulation in the nucleus, it can translocate to the cytoplasm (78). Likewise, cellular stresses that activate AMP-activated protein kinase (AMPK) such as hypoxia, hyperosmolarity and cold shock lead to the accumulation of TC45 in the cytosol (78). It seems that TC45 is exported from the nucleus to the cytosol by passive diffusion, and removal of cellular stress appears to reverse the nuclear export of TC45. It was further demonstrated that activation of AMPK using the pharmacological

inhibitor AICAR similarly induced the nuclear export of TC45 (78). The other TC-PTP isoform 48 kDa (TC48) only has 9 exons and localizes to the ER as a result of its C-terminal hydrophobic region and lack of nuclear localization sequence.

TC-PTP can be activated by ITGA1 and ITGB1, which are integrins that are involved in cell-cell adhesion and may play a role in inflammation. They have been shown to be upregulated in colorectal carcinoma and may promote cancer progression (79-82). TC-PTP has many known substrates, such as the receptor protein kinases INSR (83), EGFR (84), CSF1R (85), VEGFR2 (86) and PDGFR (87), as well as non-receptor protein tyrosine kinases JAK1 (88), JAK3 (88), STAT1 (89), STAT3 (90), and STAT6 (91).

Table 1.1: TC-PTP and PTP1B substrates

TC-PTP	**PTP1B**
JAK/STAT	**JAK/STAT**
JAK1 JAK3 STAT1 STAT3 STAT6	JAK2 TYK2 STAT6
RTKs	**RTKs**
CSF1R EGFR PDGFR INSR VEGFR2	CSF1R EGFR PDGFR INSR IGF1R

1.1.5.1 TC-PTP systemic mouse model

The first PTP mouse model generated was that of TC-PTP. A mouse model where TC-PTP was deleted systemically was generated by You-Ten and colleagues in 1997 by subcloning and embryonic stem (ES) cell transfection and implantation (92). Whole-body deletion of TC-PTP in these mice leads to severe systemic inflammation starting at one week of age which leads to runting, splenomegaly, thymic atrophy and lymphadenopathy, as well as mononuclear cellular infiltrates in the salivary glands and stomach (92). By three weeks of age, knockout mice display inflammation of the heart, gastric mucosa, salivary glands, kidneys and subcutaneous fat (77). This severe systemic inflammation ultimately leads to their death at about three weeks of age (92). These mice have an increased infiltration of macrophages in the spleen, as well as an increase in mRNA, protein and cytokine levels of iNOS, TNF-α and IFN-ɣ *in vivo* (77). TC-PTP knockout (KO) mice have many defects in hematopoietic cells, which is not surprising since TC-PTP is one of the most abundant PTP in hematopoietic cells. Firstly, these mice have defects in erythropoiesis (production of red blood cells), B cell lymphopoiesis (production of lymphocytes), and impaired B and T cell functions (92). Of particular interest for later studies, T cell development in the thymus as well as myeloid cell development in the bone marrow are unaffected.

1.1.5.2 Macrophage development and functions

Macrophages derived from these TC-PTP KO mice are more sensitive to lipopolysaccharides (LPS), which is a component of the cell wall of Gram-negative bacteria and is typically present during bacterial infections (93). The dysregulation of cytokine signaling in these mice leads to an increase production of IL-12, and this favours

an upregulation of IFN-ɣ production by T cells (77). This increase in IL-12 and IFN-ɣ production will then lead to further recruitment of immune cells such as dendritic cells, macrophages, neutrophils and T cells, which will produce even more of these and other pro-inflammatory cytokines. Furthermore, stimulation of TC-PTP KO bone marrow-derived macrophages (BMDMs) with IFN-ɣ leads to hyperphosphorylation of JAK1 compared to control mice, which leads to subsequent increase in iNOS, the primary target of IFN-ɣ signaling (85). Interestingly, one of the substrates of TC-PTP is colony stimulating factor 1 receptor (CSF-1R), which is a protein tyrosine kinase that is important for the regulation of macrophage differentiation (85). The stimulation of TC-PTP KO BMDMs with M-CSF (same as CSF1) leads to the hyperphosphorylation of the CSF-1R receptor on tyrosine 807 (Y807) (94), thus increasing its activity since Y807 is essential for signaling of the receptor (95).

1.1.6 PTP1B and TC-PTP regulate JAK/STAT signaling

PTP1B and TC-PTP are both negative regulators of the janus kinase (JAK) and signal transducer and activator of transcription (STAT) signaling pathways due to their substrate affinities. In JAK/STAT signaling pathways, cytokines are generally the main mediators and activators of the pathways. Cytokines are small proteins, such as interleukins (ILs), interferons (IFNs) or growth factors (GFs), that are secreted by cells and have an effect on other cells (93). Any given cytokine may be secreted by more than one cell type and may also activate numerous cell types through binding to their receptor. The JAK family consists of 4 members: JAK1, JAK2, JAK3 and TYK2, while the STAT family includes 6 members: STAT1, STAT3, STAT4, STAT5a, STAT5b and STAT6. The JAK/STAT proteins are crucial proteins that act as a direct link between cytokine receptors at the cell

surface and the nucleus (96). Upon binding of a cytokine to its receptor, the receptor dimerizes and brings the associated JAK molecules in close proximity. The JAKs then transphosphorylate each other on their tyrosine residues located in their activation loops, as well as phosphorylating the cytoplasmic tail of the receptor. The phosphorylated tail provides docking sites for the SH2-containing STAT molecules. Upon their recruitment and binding, they are tyrosine-phosphorylated by the JAKs which causes them to dissociate from the receptor. The phosphorylated STAT molecules subsequently form hetero- or homodimers where the SH2 domain of each STAT binds the phosphorylated tyrosine residue of the other STAT. The STAT dimer then translocates to the nucleus and binds DNA to induce the transcription of target genes, depending on the specific pathway activated (96) (**Figure 1.3**).

While PTP1B has been shown to dephosphorylate JAK2 (56-59), TYK2 (56) and STAT6 (60), TC-PTP dephosphorylates JAK1 (88), JAK3 (88), STAT1 (89), STAT3 (90) and STAT6 (91). Due to their different substrate specificities among the JAK/STAT proteins, PTP1B and TC-PTP typically regulate different pathways. For instance, inhibition of PTP1B results in increased phosphorylation and activity of both JAK2 and TYK2, and their downstream effectors STAT1 and STAT3 subsequently negatively regulate IFN-ɣ and IFN-α signaling (56). Interestingly, PTP1B can also directly bind to the cytoplasmic domain of the IL-4R, which leads to downregulation of IL-6 phosphorylation, which is downstream of the IL-4R (60). On the other hand, TC-PTP negatively regulates IL-6 and IL-4 signaling through dephosphorylation of STAT3 and STAT6, respectively (90, 91).

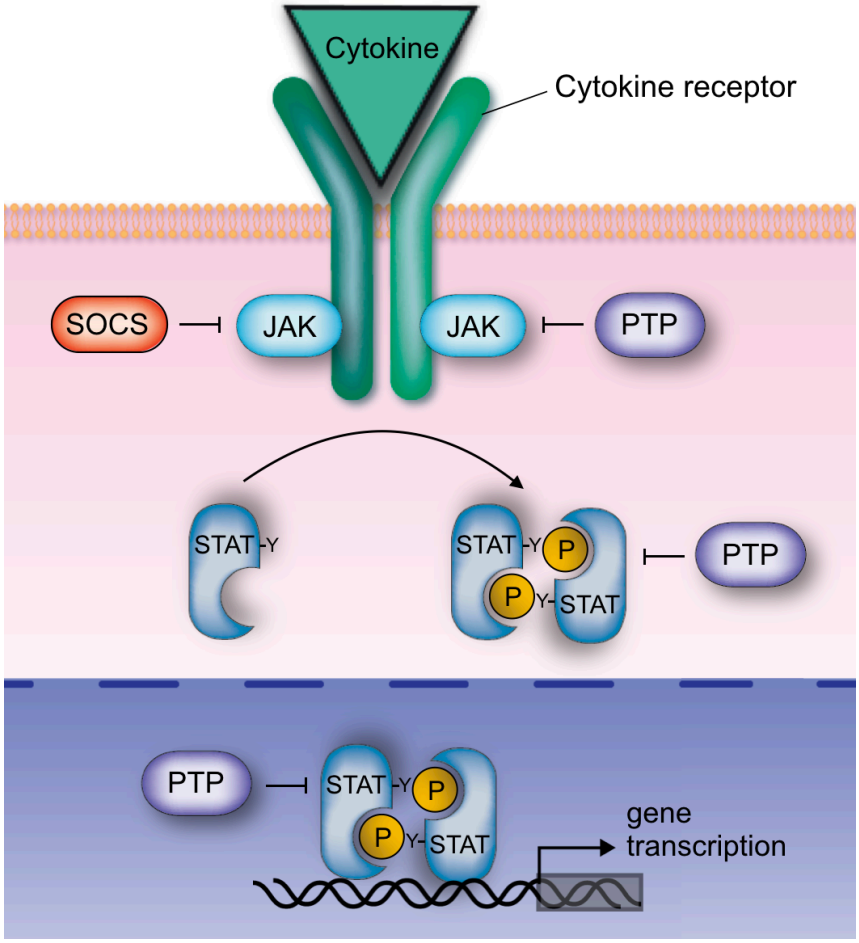

Figure 1.3: JAK/STAT signaling pathways. Upon cytokine binding to its cognate receptor, the receptor dimerizes and brings the JAK proteins in close proximity, leading to their transphosphorylation. SOCS and PTP proteins can inhibit JAK proteins. Phosphorylated JAKs recruit STAT molecules and phosphorylate them, leading to their dimerization and translocation to the nucleus to induce gene transcription. Adapted from Shuia and Lui (97).

Due to their respective substrate specificities, PTP1B and TC-PTP could theoretically effectively target the IL-6/STAT3, IL-10/STAT3, IFN-ɣ/STAT1, TNF-α/STAT3/5 and IL-4/STAT6 pathways, whereas only PTP1B could negatively regulate the IL-12/STAT4 due to its dephosphorylation of TYK2 (56).

1.1.7 Role in cancer

A multitude of PTPs have roles in different types of cancer, either as tumour-suppressors or oncogenes as a consequence of their regulation of many cellular pathways and processes. PTP1B is generally seen as an oncogenic PTP while TC-PTP more often than not appears to be acting as a tumour suppressor. In many cancers, including prostate cancer, PTP1B is detrimental as it promotes prostate cancer progression and invasion due to its dephosphorylation of key signaling proteins, thus acting as an oncogene (98, 99).

Considering that the genetic depletion of TC-PTP in mice leads to severe systemic inflammation and knowing that chronic inflammation is central to carcinogenesis (93, 100-102), it was initially postulated that TC-PTP could act as a tumour suppressor. Indeed, TC-PTP was found to be downregulated in T-cell acute lymphoblastic leukemia (T-ALL). This study demonstrated that focal biallelic or monoallelic deletion of *PTPN2* was associated with lower mRNA expression, and this was found to occur in 6% of all T-ALL patients (103). The knockdown of *PTPN2* was associated with increased proliferation and cytokine sensitivity of the leukemic cells, corroborating the tumour-suppressive role of TC-PTP (103). Interestingly, inactivation of *PTPN2* by nonsense mutations was observed in 5% of patients analyzed with peripheral T-cell lymphoma (104). In a screen of Ras-activated A549 lung adenocarcinoma cells, *PTPN2* was identified as a negative regulator of Akt, a major contributor to disease progression (105), while expression of its mRNA was decreased in human hepatocellular carcinoma lymphatic metastasis (106). In glioblastoma, TC-PTP inhibits the activity of ΔEGFR, which is a truncated form that is the most commonly found EGFR mutation in glioblastoma (84). In these glioblastoma cells

expressing the mutant ΔEGFR, TC-PTP suppresses anchorage-independent growth, *in vitro* proliferation and *in vivo* tumorigenicity, effectively acting as a tumour-suppressor (84). Contrary to its effect in most cancer types, TC-PTP seems to act as an oncogene in B cell lymphomas since it is overexpressed in some cell lines of activated B-cell-like diffuse large B cell lymphomas (91). TC-PTP is similarly overexpressed in other human and murine B cell lymphomas (107). Additionally, in murine MYC-driven B cell lymphomas, TC-PTP expression was correlated with the overexpression of the MYC transcription factor, while its knockdown severely decreased murine B cell lymphoma cell proliferation and abolished tumour maintenance *in vivo* (107), emphasizing its potential oncogenic role in this type of cancer in cooperation with MYC. Due to its involvement in various cancers and inflammation, and considering that TC-PTP is activated by ITGA1 and ITGB1, we postulated that TC-PTP could play a role in colorectal cancer.

1.2 Colorectal cancer

In Canada, the most common type of death is due to cancer, and of all cancer types colorectal cancer (CRC), also known as colon cancer or bowel cancer, is the third most common type based on both incidence and mortality (108). As of 2015, colorectal cancer is the third deadliest cancer worldwide (109). The incidence of colorectal cancer has generally decreased over time, most likely due to the increase in CRC screening which allows to detect precancerous lesions, called polyps (108). However, it is interesting to note that incidence rates have decreased in older adults, but increased in adults younger than 50 years old (108).

Colorectal cancer is a malignant tumour that begins in the cells of either the colon or the rectum. The most common type of CRC, comprising 95% of all cases, is called

adenocarcinoma and refers to cancer that begins in gland cells that line the colon or rectum. The colon is composed of four main segments: the ascending colon, transverse colon, descending colon and sigmoid colon (110) (**Figure 1.4**). These are each composed of four layers: the mucosa (innermost lining of the colon and rectum), submucosa (layer of connective tissue that surrounds the mucosa), muscularis propria (thick layer of muscle that surrounds the wall of the colon and rectum) and serosa (outer layer of the colon). Of particular interest is the mucosa, which is itself composed of a thin layer of epithelial cells, called the epithelium, followed by a layer of connective tissue called the lamina propria, and finally a thin layer of muscle, called the muscularis mucosa (93, 110). As the innermost layer of the colon, the mucosa is essential in maintaining the epithelial barrier to protect the colon from invading microorganisms such as bacteria.

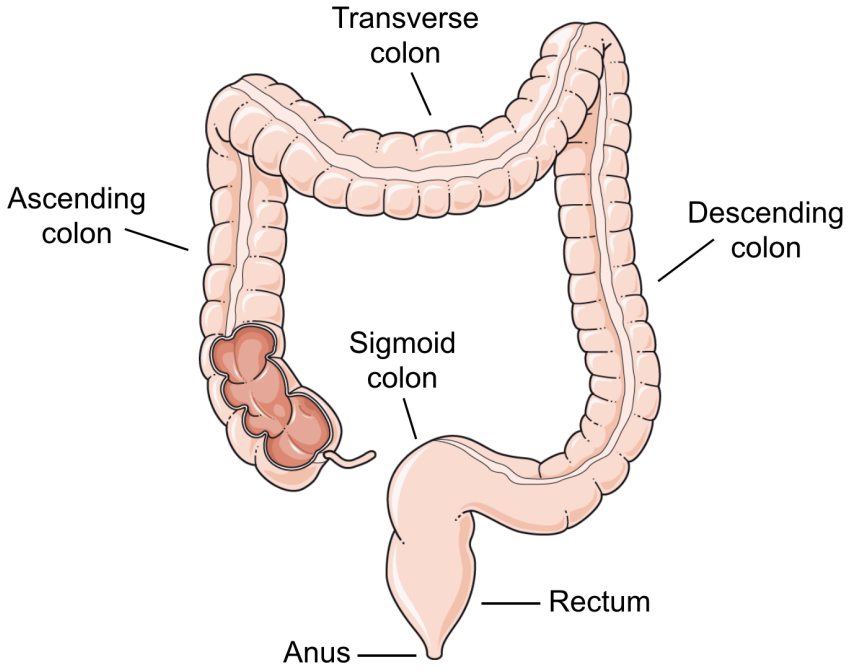

Figure 1.4: Structure of the colon. The colon, or large intestine, is composed of four main segments: the ascending colon, transverse colon, descending colon and sigmoid colon, as well as the rectum and the anus. Figure modified from SMART (111).

1.2.1 Risk factors and genetic mutations

The development and progression of colorectal cancer is associated with several modifiable risk factors, including obesity and physical inactivity, consumption of red and processed meat, smoking, alcohol consumption and inflammatory bowel diseases (IBDs) such as Crohn's disease (108, 112, 113). Having hereditary syndromes such as familial adenomatous polyposis (FAP), or a family history of polyps or CRC may also be risk factors for the development of colon adenomas (108, 112, 113).

In addition to these risk factors, many genetic mutations have been established to be associated with CRC. These include *DCC*, *KRAS* and *BRAF* genetic mutations. *DCC* is a tumour suppressor that is often missing in colorectal cancer cells as it is deleted in about 30% of CRC tumour cells (113). The absence of *DCC* has also been linked with metastases and resistance of patients to chemotherapy (108). *KRAS* has been found to be mutated in 30-50% of CRC tumours, and this mutation typically correlates with lack of responsiveness of the cancer cells to targeted therapy drugs. Finally, mutations in *BRAF* may lead to more aggressive cancer cells. Patients with BRAF mutations, representing about 5-10% of CRC tumours, typically have the worst prognosis (108, 113). Fortunately, these mutations alone are not sufficient to induce the development of colorectal cancer. CRC typically requires a half dozen or more mutations to initiate the formation of a cancer cell (113). For example, after the development of a polyp due to several genetic mutations, the mutation of one copy of the *KRAS* proto-oncogene transforms the polyp into an adenoma. To progress further to cancer, both alleles of specific genes need to be mutated in the adenoma (113). Colorectal cancer is, without a doubt, a multistep process that results from several risk factors as well as many genetic mutations. There are other

risk factors and characteristics that can lead to the initiation and progression of not only colorectal cancer, but many other cancer types as well. These are termed “hallmarks of cancer”.

1.2.2 Hallmarks of cancer

In 2000, Hanahan and Weinberg published an eminent paper summarizing the now well-established “Hallmarks of Cancer”, with an updated version published a decade later (50, 114). The initial model includes six hallmarks that they deemed based on a plethora of studies to be characteristic of many cancer cell types: evading apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, tissue invasion and metastasis, and sustained angiogenesis (114). The most recent model contains two novel hallmarks as well as two enabling characteristics that will facilitate the acquisition of the hallmarks (**Figure 1.5**). The two emerging hallmarks of cancer are reportedly involved in the pathogenesis of some, if not all, cancers. One is the ability of cancer cells to reprogram their cellular metabolism in order to support proliferation, while the other is the cells’ ability to evade destruction by immune cells, particularly from macrophages, T cells, B cells, and natural killer cells (50). The two enabling characteristics of cancer progression are genomic instability and tumour-promoting inflammation. Unfortunately for the host, induction of inflammation by innate immune cells such as macrophages to combat infections and perform wound healing can result in their unintentional support of several hallmarks of cancer, thus enabling cancer progression (50).

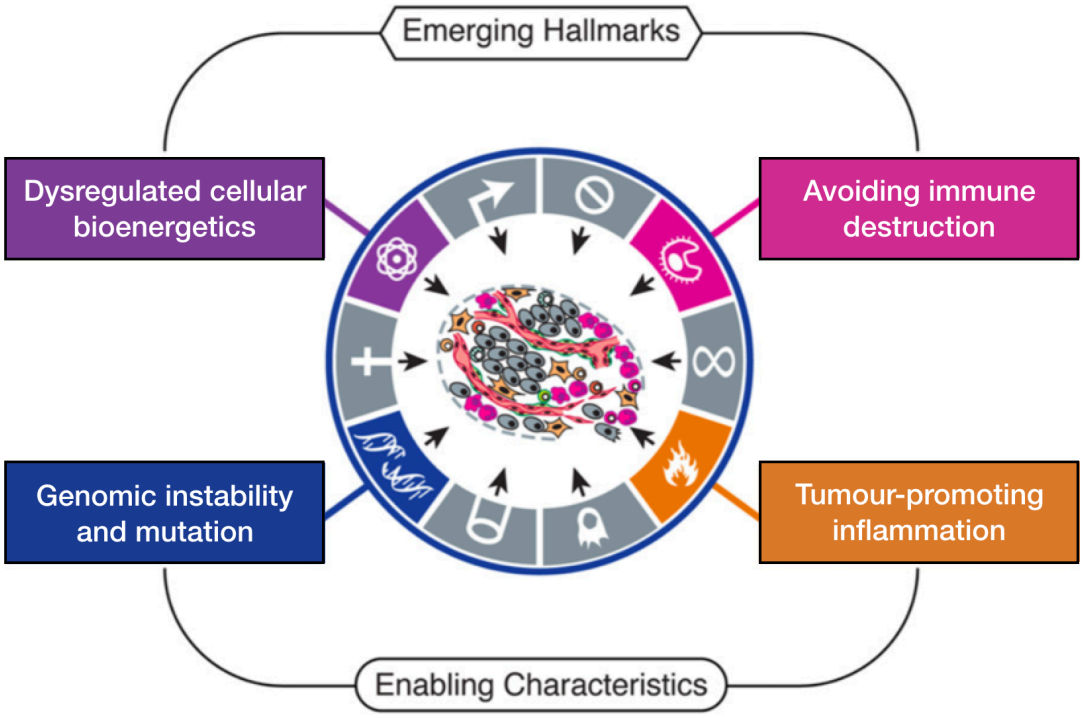

Figure 1.5: Emerging hallmarks of cancer. To the already well-established hallmarks of cancer, two new hallmarks of cancer have been proposed, as well as two enabling characteristics. Dysregulated cellular bioenergetics and avoiding immune destruction are emerging hallmarks of cancer that are involved in the pathogenesis of some, if not all, cancers. The two enabling characteristics that promote carcinogenesis through acquiring the hallmarks of cancer are genomic instability and mutation, and tumour-promoting inflammation. Figure adapted from Hanahan and Weinberg (50).

1.2.3 Inflammatory bowel diseases

Indeed, tumour-promoting inflammation has been established as a hallmark of cancer, with colorectal cancer being the prototype for cancer resulting from chronic inflammation (50). Chronic inflammation of the colon is characteristic of inflammatory bowel diseases (IBDs), which is a risk factor for the development of CRC (115, 116). There are two main types of IBDs: ulcerative colitis (UC), which is limited to the colon and where the mucosa is majorly involved, and Crohn's disease (CD), which can affect any segment of the gastrointestinal tract from the mouth to the anus. CD differs from UC by

its “skip lesions” form, which was thus named because areas of inflammation in the gastrointestinal tract are generally not continuous throughout (117). Several studies have demonstrated that increased duration and severity of inflammation increases the risk of CRC development by 60% in IBD patients (118). It is resulting from the stimulation and recruitment of immune cells at the site of inflammation that chronic inflammation develops into the appropriate environment for tumour initiation and progression (119).

There are many cytokines and chemokines produced in IBD patients that lead to the activation and/or recruitment of immune cells. For instance, there is an increase in IL-6 and IL-6 receptor (IL-6R) in IBD patients, and this leads to the colonization of the lamina propria by T cells, which play a role in the progression of chronic inflammation (120). Similarly, IL-10 is an anti-inflammatory cytokine that plays an important role in colitis-associated colorectal cancer (CAC). In mouse studies, IL-10 deficiency has been shown to lead to spontaneous colitis (121), while patients with a mutant IL-10 which abrogates signaling had more aggressive tumour progression (120). On the other hand, TNF-α produced by immune cells in IBD patients promotes tumorigenesis by inducing apoptosis in epithelial cells (122). In mice, deficiency of TNF-α results in a decrease in the infiltration of inflammatory immune cells, in cytokine production and in mucosal harm which, in turn, correlates with a slowing down of tumour progression (120). More specifically, the presence of TNF-α secreted by immune cells at the site of inflammation, such as is the case in the colon of IBD patients, induces the activation of monocytes, macrophages and neutrophils, and subsequent production of ROS, leading to exacerbation of tumour progression (122). Hence, identifying proteins and understanding the mechanisms that

promote dysregulated cytokine signaling and production will be essential in trying to dampen inflammation to reduce colorectal cancer progression.

1.2.3.1 Role of TC-PTP in colitis

In 2007, a genome-wide study performed by Wellcome Trust Case Control Consortium (WTCCC) identified a potential role for TC-PTP in inflammatory bowel diseases (IBDs) (123). In this study, single nucleotide polymorphisms (SNPs) were screened from patients that had one of three types of autoimmune disorders: Crohn's disease, rheumatoid arthritis or type I diabetes. This study discovered two SNPs that correlated with CD: rs2542151 and rs1893217. rs2542151 is located 5.5 kb downstream of the 3' end of the *PTPN2* transcript and it was found to be strongly related to CD. rs1893217 is a second SNP that is located on intron 7 of *PTPN2* and is also related to CD (123, 124). The potential role of TC-PTP in IBDs was also described in an Italian cohort of patients (125). Moreover, another study has demonstrated that TC-PTP mRNA and protein (126) levels were higher in patients with CD compared to control patients, further validating the potential role of TC-PTP in regulating inflammatory bowel diseases such as Crohn's disease. The increase of TC-PTP in CD patients may seem counter-intuitive since TC-PTP knockout mice develop chronic inflammation, a risk factor for IBDs. This would have to be further studied, but could be due to the upregulation in pro-inflammatory cytokines commonly present in CD patients, such as TNF-α and IFN-ɣ. Studies have demonstrated that both TNF-α (126) and IFN-ɣ (127) stimulation induces the mRNA expression and activity of *PTPN2* in intestinal epithelial cells (IECs).

1.2.4 Macrophages

As we now know, tumour-promoting inflammation is a well-established hallmark of cancer. Macrophages are cells that are heavily involved in the induction of inflammation, particularly in the case of colorectal cancer (50). Macrophages can exist as resident macrophages which are localized in specific tissues or be differentiated from circulating monocytes following an insult, such as a wound or tumour cell differentiation (93, 128). Macrophages are the predominant type of phagocytic cell and, along with neutrophils, they serve as the first line of defense in innate immunity (93, 128). They secrete a plethora of cytokines and chemokines, including IFN-ɣ, IL-6, IL-10, IL-12, TNF-α, CXCL8 and M-CSF, that function in activating other immune cells and attracting them to the site of injury, thus initiating an inflammatory cascade (93, 128, 130-134). Macrophages can also promote the activation of more macrophages as a result of this cytokine production. The cytokines produced will form a gradient that will recruit macrophages and other immune cells to the source of cytokine release in a paracrine effect (93, 102). The PTP transcriptome in immune cells has been well characterized, with 76 PTP-encoding genes found to be expressed in macrophages. 64 of these PTPs are expressed in all immune lineages, including dendritic cells, B cells, T cells and neutrophils, while the other 12 PTPs were only expressed in some lineages such as immature dendritic cells (128, 135).

1.2.4.1 Macrophage polarization

Beyond promoting inflammation and recruiting immune cells to the site of injury, macrophages also have important functions in promoting an anti-inflammatory response. They can secrete anti-inflammatory cytokines and chemokines that can either dampen pro-inflammatory responses or directly stimulate other immune cells (93). There are two

main types of immune phenotype in macrophages: pro-inflammatory macrophages (also called classically activated) are referred to as M1 macrophages, while anti-inflammatory macrophages (also called alternatively activated) are known as M2 macrophages (129-132). It is now well understood that macrophages are very dynamic and can switch from one polarization phenotype to another, depending on the cues from the microenvironment and tumour cells. This allows them to adapt and perform the necessary functions as dictated by the particular immune response at a given point. Of note, recent studies have demonstrated that there are more than M1 and M2 macrophages, but rather there is a gradient of immune phenotype and functions that vary from the extremes of pro-inflammatory to anti-inflammatory, with distinct phenotypes in between (133, 134). Nonetheless, for the purpose of this study we will focus on M1 and M2 macrophages only, keeping in mind that macrophages have a very high plasticity. M1 macrophages favour an anti-tumour Th1 response and also function in the killing of parasites. IL-12 and TNF-α are two cytokines characteristic of M1 macrophages, as they will secrete them and induce a pro-inflammatory immune response (135, 136). On the other hand, M2 macrophages favour tumour progression through induction of a Th2 response. These macrophages promote angiogenesis and have a high expression and production of IL-10 and arginase 1 (130, 136, 137) (**Figure 1.6**).

Various PTPs have been shown to regulate the polarization of macrophages. PTPN22 was shown to act as a positive regulator of anti-inflammatory M2 macrophage polarization upon IL-4 and IL-13 stimulation in a STAT6-dependent manner (138). On the other hand, SHP-1 and PTP1B have been established as negative regulators of M1 macrophage polarization induced by LPS by regulating the NF-κB signaling pathway

(138). These studies shed light on the potential of PTPs in reprogramming macrophages towards the anti-tumour M1 phenotype.

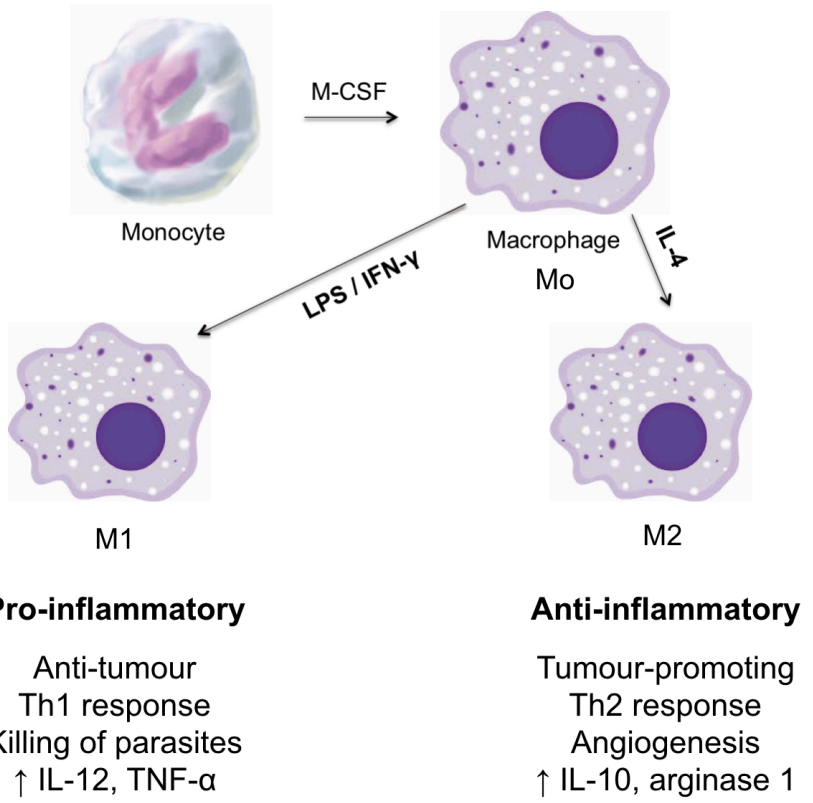

Figure 1.6: Differentiation and polarization of macrophages. Monocytes harvested from the bone marrow can be differentiated into macrophages with M-CSF. These naive macrophages (Mo) can then be polarized into pro-inflammatory (M1) macrophages using LPS and IFN-ɣ or towards anti-inflammatory (M2) macrophages using IL-4.

1.2.4.2 Macrophage regulation

The functions of macrophages can be regulated by several classes of enzymes, including receptor tyrosine kinases (RTKs) and protein tyrosine phosphatases (PTPs). To date, several PTPs have been shown to negatively regulate macrophage differentiation and activation, notably PTP1B, TC-PTP, SHP-1, SHP-2 (gene name *PTPN11*) and PTPN22, due to their dephosphorylation of RTKs and other signaling substrate (138). SHP-2 has been demonstrated to negatively regulate IL-4-induced macrophage

activation through regulation of the JAK1/JAK3-STAT6 signaling pathway (138, 149). Similarly, PTPN22 negatively regulates IFN-ɣ-induced macrophage activation through the regulation of JAK1/JAK2-STAT1 signaling pathways (138).

PTPs can also regulate macrophage functions by modulating cytokine signaling. CD45 negatively regulates cytokine signaling in macrophages upon IFN-α stimulation through a TYK2-STAT1/STAT3 signaling pathway (138, 150). SHP-1 is another IFN-α-induced regulator of JAK1/STAT signaling in macrophages (138, 151). One report demonstrated that myeloid-specific deletion of PTP1B protected mice against LPS-induced inflammation due to the systemic increase in IL-10 and STAT3 hyperphosphorylation (139). The induction of the IL-10/STAT3 signaling pathway is known to cause an increased expression of the suppressor of cytokine signaling 3 (SOCS3), which acts as a negative feedback regulator of the pro-inflammatory IL-6/STAT3 signaling pathway (138, 153). Consequently, SOCS3 is thought to be a major player in reducing inflammation through downregulation of pro-inflammatory cytokine signaling, and this is particularly true in activated macrophages (140). As such, by inhibiting PTP1B in myeloid cells, SOCS3 may be upregulated upon the stimulation of IL-10 and thus negatively regulate pro-inflammatory cytokine signaling, acting as a critical dampening checkpoint for the pro-inflammatory response in macrophages (138).

Interestingly, it was found that stimulating murine peritoneal macrophages with LPS leads to an alteration of PTP gene expression. For instance, *Ptprj* and *Mtmr7* were found to be increased upon LPS stimulation, whereas *Dusp27* and *Cdc25b* were found to be decreased (138, 135, 155). These studies emphasize that regulation and activity of PTPs could be controlled by pro-inflammatory stimuli. Considering their various roles in

inhibiting macrophage differentiation, activation and polarization, PTPs would be ideal targets for macrophage-based cancer immunotherapy.

1.3 Metabolism

Cellular metabolism includes a plethora of pathways that a cell employs for one of three main purposes: 1) convert food and fuel to energy to perform cellular processes (bioenergetic), 2) convert food and fuel into building blocks for protein, lipid, nucleic acid and carbohydrate synthesis (biosynthesis), and 3) elimination of nitrogenous wastes (141). The two predominant metabolic pathways in cells are glycolysis and oxidative phosphorylation. Both of these pathways are used to generate adenosine triphosphate (ATP), the main form of energy in the cell (141). The amount of ATP in a cell is usually only sufficient to supply energy needs for one or two minutes, so it is constantly being hydrolyzed and regenerated. Cells and tissues with a high metabolic activity can suffer greatly from oxygen deprivation, also called hypoxia, since considerably less ATP is being produced during that time. Hypoxic cells typically reprogram they metabolism in order to generate ATP through glycolysis (141). As such, it is essential that cellular metabolism be properly regulated.

1.3.1 Glycolysis

In eukaryotes, glycolysis is the main pathway of glucose catabolism and occurs in the cytosol in both anaerobic and aerobic conditions. In normal cells, anaerobic glycolysis is preferred and has a central metabolic role in generating energy and metabolic intermediates to feed into other pathways (141). The glycolytic pathway utilizes glucose as its major fuel and consists of 10 enzymatic reactions, the first five of which require an

energy investment, while the last five reactions generate energy in the form of ATP (141). Anaerobic glycolysis leads to the production of pyruvate and a small amount of ATP. In absence of oxygen, pyruvate will be fermented to lactate (141). In aerobic conditions, when oxygen is present, pyruvate will instead be further metabolized and undergo oxidative metabolism through the citric acid cycle (CAC) and oxidative phosphorylation (OXPHOS). Prior to entering the CAC, pyruvate is converted to acetyl-coenzyme A (acetyl-CoA).

Other pathways of glucose catabolism include the pentose phosphate pathway (PPP), which competes with glycolysis for glucose-6-phosphate, the product of the first reaction of glycolysis catalyzed by hexokinase. The PPP converts glucose to other sugars such as nicotinamide adenine dinucleotide phosphate (NADPH) and ribose-5-phosphate which can be used for energy and nucleotide biosynthesis, respectively (141). Rapidly proliferating cells such as cancer cells typically have a high activity of PPP enzymes due to the role of NADPH in cell survival in conditions of stress as well as the role of ribose-5-phosphate in nucleic acid synthesis (141). In addition, in highly aerobic cells or tissues, glucose can be generated from lactate by gluconeogenesis to upregulate pathways involved in glucose catabolism. Consequently, it is important to consider alternative pathways of glucose catabolism when investigating cellular metabolism, particularly glycolysis.

1.3.2 Oxidative phosphorylation

In eukaryotes, OXPHOS occurs in the mitochondria and is performed in aerobic conditions. This metabolic pathway generates ATP through the oxidation of nutrients (141). Compared to anaerobic glycolysis, it is highly efficient at producing ATP, with about

an 18-fold increase in ATP generated. Upon conversion of pyruvate to acetyl-CoA in the cytoplasm, acetyl-CoA enters the CAC, also called tricarboxylic acid cycle (TCA) in the mitochondrial matrix. The complete oxidation of acetyl-CoA by enzymes of the CAC leads to the production of CO_2, reduced electron carriers including NADH and $FADH_2$, and a small amount of ATP (141). The two reduced electron carriers are reoxidized by going through the electron transport chain (ETC), and it is this last step of aerobic cellular respiration that generates most of the energy used to synthesize ATP.

1.3.2.1 The electron transport chain (ETC)

The ETC, also called respiratory chain, occurs at the inner mitochondrial membrane (IMM) and consists of a series of oxidation and reduction reactions, with electrons being passed along a series of electron carriers (141). Respiration requires an intact IMM to ensure the passage of electrons down the ETC. The energy released by this process is used to transport protons across the IMM and thus to generate a proton gradient, which provides the driving force for ATP synthesis. If the IMM is disrupted, the electron flow will be uncoupled from ATP production. Electrons will still flow and oxygen will still be consumed, but the synthesis of ATP will be inhibited (141). The ETC is composed of five multiprotein enzyme complexes (**Figure 1.7**). Complex I and II are NADH dehydrogenase and succinate dehydrogenase. They receive electrons from the oxidation of NADH and succinate, respectively, and pass them along to the lipid-soluble electron carrier coenzyme Q (CoQ). Complex III is cytochrome c reductase and, as its name implies, catalyzes the transfer of electrons from the reduced CoQ to cytochrome c (Cyt c). Cyt c is a soluble protein that acts as an electron carrier and is mobile in the intermembrane space of the mitochondria. Complex IV is cytochrome c oxidase and it catalyzes the

oxidation of cytochrome c. The energy released from the reactions performed by complexes I, II, III and IV is used to pump protons from the mitochondrial matrix to the intermembrane space, thus creating a proton gradient. The last complex, Complex V, is ATP synthase. Protons re-enter the mitochondrial matrix through a specific channel in the ATP synthase. The free energy stemming from this process drives the synthesis of ATP, hence its name (141). A variety of chemical compounds can be used to inhibit different complexes of the ETC, notably rotenone (complex I inhibitor), antimycin A (complex III inhibitor), oligomycin (complex V inhibitor) and carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP, membrane uncoupler) (**Figure 1.7**).

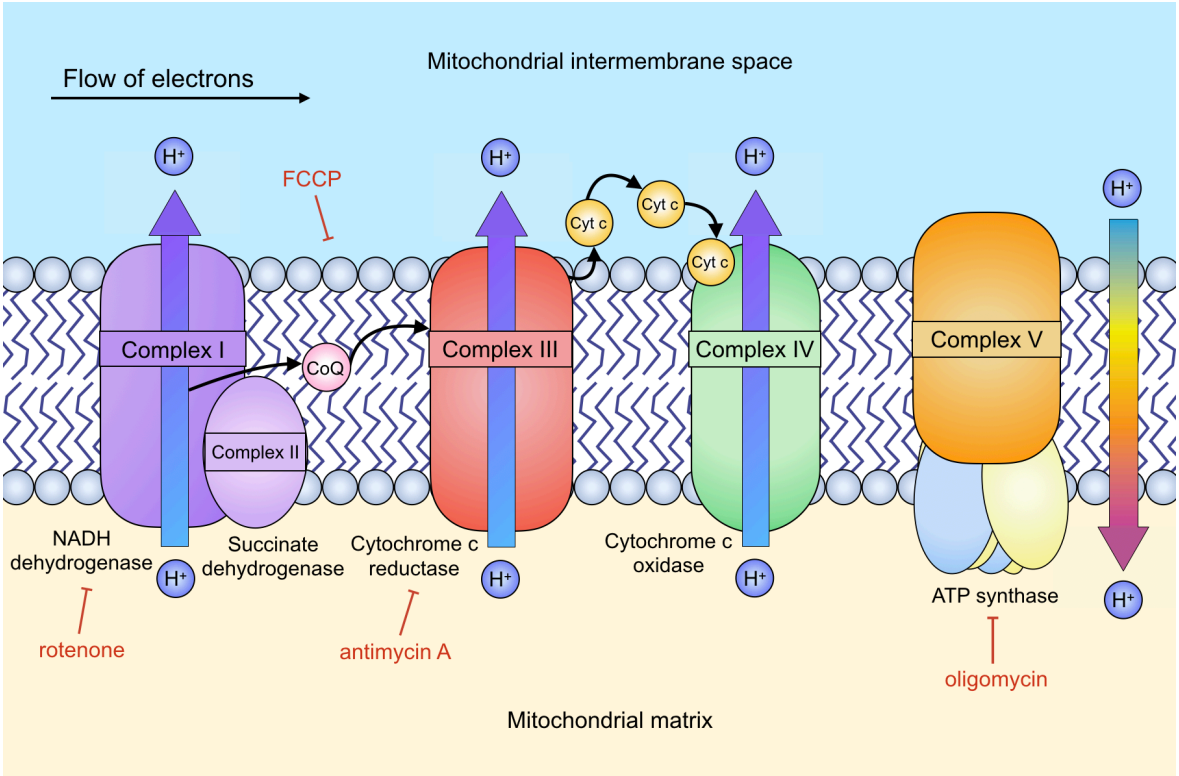

Figure 1.7: Electron transport chain. Different complexes of the ETC can be inhibited by using different compounds. Oligomycin inhibits ATP synthase (Complex V) and thus ATP production. FCCP permeabilizes the membrane and uncouples the proton gradient from ATP synthesis. Rotenone inhibits NADH dehydrogenase (Complex I), while antimycin A inhibits cytochrome c oxidase (Complex III), both of which disrupt the proton gradient and inhibit ATP synthesis. Figure adapted from Dorn (142).

In normal mitochondria, to prevent the wasteful oxidation of metabolites, electron flow through the ETC only occurs when there is a requirement for ATP synthesis. On the other hand, ATP synthesis is dependent on electron flow from substrates to oxygen, the final electron acceptor in the ETC (141).

1.3.2.2 Role of STAT proteins in the mitochondria

It has long been known that phosphorylation events are not restricted to the cytosol and nucleus. Two decades ago, several studies demonstrated that there were a plethora of phosphoproteins in the mitochondria, several of which are part of OXPHOS or the ETC (143, 144). For instance, Struglics and colleagues were the first to show that two subunits from the ATP synthase of the ETC were phosphorylated, one from the Fo region and one from the F1 region, and that these phosphorylation events could be responsible for the coupling of the two subunits and the activity of the ATP synthase (143). Moreover, several RTKs were shown to be present in the mitochondria and regulate mitochondrial dynamics through phosphorylation events (145-149). Knowing that there are several mitochondrial phosphoproteins and that many RTKs are localized in the mitochondria, it would be reasonable to assume that PTPs would also be present in the mitochondria, as well as some JAK or STAT proteins that they regulate.

Indeed, several reports have established the presence of different STAT proteins in the mitochondria, mostly STAT1 and STAT3. A first study demonstrated that apart from its well-known role as a transcription factor in the nucleus, STAT3 could also localize to the mitochondria and specifically regulate metabolic functions that could promote Ras-dependent malignant transformation (150). Localization of STAT3 to the mitochondria

seems to require phosphorylation on Ser727, although the kinase responsible for its phosphorylation is not known at this point. Interestingly, loss of mitochondrial STAT3 (mitoSTAT3) resulted in a 50% decrease in ATP levels as well as impairment of complexes II and V of the ETC. As such, the authors demonstrated that localization of STAT3 to the mitochondria impairs mitochondrial function as well as ATP production (150). At the same time, another study reported that loss of mitoSTAT3 can also downregulate the activity of complexes I and II of the ETC, and further discovered that STAT3 is likely present at the IMM, where the ETC is present (151). A recent study by Meier et al. provided further information regarding the functions of STAT3 in the mitochondria. They found that presence of STAT3 in the mitochondria was dependent on certain stimuli such as the presence of ROS or cytokines (149). Stimulation of cells with H_2O_2 or IL-6 led to a timely decrease in STAT3 present in the mitochondria. They confirmed previous studies regarding the importance of Ser727 phosphorylation for STAT3 mitochondrial localization, as this phosphorylation was essential for the recovery of STAT3 in the mitochondria (149, 152). They proposed that mitoSTAT3 is a sensor for metabolic stresses, leading to modulation of mitochondrial respiration upon its activation (149).

Similarly, although fewer studies have been conducted, STAT1 has also been found in the mitochondria. One report demonstrated that STAT1 localizes to the mitochondria and negatively modulates mitophagy, which is autophagy of mitochondria, following a stress simulated by ischemia/reperfusion injury. They observed an increase in death of mitochondria in absence of STAT1 (153). Mitochondrial STAT1 (mitoSTAT1) was also found to inhibit mitochondrial biogenesis, as mitochondria from the liver of STAT1

knockout mice were increased in number and demonstrated defects in the ETC (154). Whether these effects are due to downregulation of STAT1 itself or to decreased expression of STAT1 target genes remains to be established.

These various studies emphasize a role for STAT proteins, particularly STAT1 and STAT3, in regulating mitochondrial functions. More specifically, they are positive modulators of mitochondrial respiration through their regulation of complexes of the ETC. It would be crucial to determine the upstream signaling pathways that modulate STAT1 and STAT3 phosphorylation and localization to the mitochondria to better understand how, and why, they modulate mitochondrial respiration.

1.3.3 The Warburg effect

In most cells, approximately 90% of the molecular oxygen consumed is utilized for OXPHOS for the generation of ATP. The rest of the ATP is typically generated through the glycolytic pathway. However, the understanding of mitochondria usage by cancer cells has been revolutionized more than 60 years ago. In 1956, Otto Warburg was the first to describe what is now widely referred to as the “Warburg Effect”, which is the discovery that cancer cells preferentially undergo glycolysis as opposed to oxidative phosphorylation, even in the presence of ample oxygen (155). This metabolic switch is referred to as aerobic glycolysis and is considered a hallmark of cancer. This has been summarized in Hanahan and Weinberg’s “Hallmarks of Cancer” as the capability of cancer cells to “reprogram their cellular metabolism in order most effectively support neoplastic proliferation” (50). The mitochondria do remain functional and some OXPHOS still occurs in cancer cells, but most of the glucose is going through the glycolytic pathway and being converted to lactate (156). One would wonder why cancer cells would favour

this low energy-generating glycolytic pathway as opposed to OXPHOS, which produces about 18 times as much ATP (114, 141). We are now beginning to uncover that cancer cells have important metabolic needs that far exceed the requirement for ATP. Glucose can be used not only to generate ATP through glycolysis, it is also essential to produce biomass that will allow for very rapidly proliferating cells such as cancer cells to grow and divide. The main two metabolites that are catalyzed in mammalian cells are glucose and glutamine. Hence, these need to be used not only for ATP production, but also to be diverted to macromolecular precursors such as acetyl-CoA for the generation of fatty acids, glycolytic intermediates for the generation of non-essential amino acids, and ribose for the generation of nucleotides (156). The Warburg effect may be the underlying driving mechanism of many cancer cells due to its regulation of other hallmarks such as inflammation.

1.3.3.1 Regulators of the Warburg effect

Several regulators of the Warburg effect have been established to date, some of which are oncogenes while others are tumour suppressors. A few of the most well-known ones are LKB1, AMPK and p53. LKB1, or liver kinase B1, is a serine/threonine kinase that is a well-known tumour suppressor and is often inactivated in cancer. Of note, it is its inactivation that is associated with tumour progression, not its activation (157, 158). In the cell, LKB1 provides a metabolic response to energetic stresses, and its inhibition can lead to the development of many cancer hallmarks (50). One of its downstream targets is AMP-activated protein kinase (AMPK). Upon energy stress, it directly phosphorylates AMPK and leads to its activation (159-161). Loss of LKB1 has recently been shown to promote metabolic reprogramming of cancer cells (157). LKB1-deficient cells demonstrated an

upregulation of both glycolysis and flux into the TCA cycle, as well as an increase in glucose and glutamine metabolism (157). Hence, it appears that inactivation of LKB1 completely reprograms the metabolism of cancer cells, promoting both OXPHOS and glycolysis and thus perhaps cancer cell growth.

AMPK is another serine/threonine kinase that is a tumour suppressor. It is a well-established master regulator of energy homeostasis and metabolism. It is downstream of LKB1 and can be activated by this kinase, but it can also be activated when there is a low [AMP]/[ATP] ratio. That is to say that when ATP levels are low in the cell, AMPK becomes activated and will favour pathways that produce ATP, such as OXPHOS, while inhibiting pathways that require it for their function. Of note is that while AMPK activation leads to an increase in glucose uptake, this event will eventually promote OXPHOS as opposed to glycolysis due to the requirement of the cell for larger amounts of ATP (156). AMPK can also phosphorylate and activate mitochondrial proteins such as PGC-1α and p53, further ensuring increased mitochondrial functions (162). AMPK has recently been demonstrated to be a negative regulator of the Warburg effect. Loss of AMPK promoted the shift from mitochondrial respiration to aerobic glycolysis and increased biomass accumulation (163). Like LKB1, presence of AMPK would not be beneficial for cancer cells.

p53 is another tumour suppressor and is one of the most frequently mutated genes in cancer. Its inactivation has been demonstrated to control metabolic genes and alter the utilization of glucose (156). More specifically, it has been shown to modulate the Warburg effect through regulation of synthesis of cytochrome c oxidase 2 (SCO2), which controls cytochrome c oxidase complex (complex IV) of the ETC (164). Matoba and colleagues

established that p53-deficient colorectal cancer cells downregulate the expression of SCO2 and by doing so favour a switch from mitochondrial respiration to glycolysis, demonstrated by a decrease in oxygen consumption and an increase in ATP generation from lactate, the end-product of glycolysis (164). This report provided insight into how a tumour suppressor such as p53 that is inactivated in cancer may promote tumourigenesis by decreasing the cellular dependency on oxygen and promoting the Warburg effect, perhaps permitting rapid growth in hypoxic environments (164).

Considering that these tumour suppressors and some oncogenes have been reported to be negative or positive regulators of the Warburg effect, other such proteins should be assessed for their ability to promote cancer progression by favouring the Warburg effect. Such targets are the PTPs, which is a family consisting of over one hundred members that have been shown to act as both oncogenes and tumour-suppressors, as well as being master regulators of STAT proteins that can localize to the mitochondria and regulate mitochondrial respiration.

1.3.3.2 Experimental approach to assess the Warburg effect

A great experimental tool has been designed to measure cellular respiration. It is called the Seahorse assay and consists in plating the desired cells, for instance colorectal cancer cells, in a microplate and after a rest period injecting drugs to inhibit different components of the ETC. To measure mitochondrial respiration performed by the cells, the drugs oligomycin, FCCP, rotenone and antimycin A are used, in that order (**Figure 1.7**). Oligomycin inhibits the ATP synthase (Complex V) of the ETC by inhibiting the proton gradient, leading to inhibition of ATP production and reduction of the electron flow through the ETC (141). This disruption leads to a decrease in oxygen consumed by

the cells, and this is measured using probes, which come in close proximity to the cells and measure the oxygen consumption rate (OCR) from the extracellular media. FCCP is an H^+ ionophore that chemically uncouples electron flow from ATP production by permeabilizing the IMM to protons, thus disrupting the proton gradient (141). Electrons continue to pass through the ETC and oxygen is still being consumed by the cells, but ATP is no longer being synthesized due to the absence of the proton gradient. In order to maintain the membrane potential, the mitochondria increase the flow of electrons through the ETC, and as a result the oxygen consumption is at maximal capacity and reflects how cells react to increased ATP demands, such as in conditions of stress like hypoxia or nutrient deprivation (50). Rotenone inhibits the enzyme NADH dehydrogenase (Complex I) by blocking the electron flow from NADH to CoQ. Its inhibition dysregulates ATP production as well as oxygen consumption (141). Antimycin A inhibits the enzyme cytochrome c reductase (Complex III) of the ETC and this disrupts the formation of a proton gradient across the IMM, resulting in inhibition of the electron flow through the ATP synthase and consequently inhibiting ATP production. After injection of each of these drugs in the extracellular media, the instrument measures the OCRs of the cells which are then used to establish a mitochondrial respiratory profile.

Glycolysis can also be measured with this system using glucose, oligomycin and 2-DG, an analog of glucose that cannot undergo further glycolysis. Glucose will stimulate glycolysis while 2-DG will inhibit it by competitively preventing glucose from being metabolized through the glycolytic pathway. Injection of these drugs in the extracellular media of cells will differentially regulate glycolysis, and this can be measured by determining the pH of the extracellular media. Given that lactate is an end product of the

glycolytic pathway, its production will render the extracellular media more acidic due to concomitant H^+ production and can be measured as the extracellular acidification rate (ECAR) of the cells.

There are several other techniques that can be used to measure the Warburg effect. To measure the glucose uptake and lactate production representing glucose catabolism indicative of glycolysis, a BioProfile Analyzer could be used to measure these nutrients and metabolites. Gas chromatography mass spectrometry (GC-MS) metabolite profiling could be done to follow the metabolites of glycolysis and the TCA cycle in TC-PTP-deficient colorectal cancer cells. Finally, stable isotope tracer analysis (SITA) could subsequently be performed using mitochondrial inhibitors and ^{13}C-pyruvate as a substrate to obtain a complete targeted profile of the organic acid intermediates of the TCA. Together, these methods would allow us to have a good picture of the relative usage of glycolysis and respiratory pathways by the cells. However, the Seahorse assay is one of the best cost-efficient methods of assessing the Warburg effect in cancer cells and is a starting point in our studies.

CHAPTER 2

A HUMAN PHOSPHATOME METABOLIC SCREEN IDENTIFIES TC-PTP AS A REGULATOR OF COLORECTAL CANCER BIOENERGETICS

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2.1 Introduction

Mitochondria are known as the “powerhouse of the cell” because they are normally responsible for the production of more than 90% of cellular ATP, the cell’s energy, and they are a major source of reactive oxygen species (ROS) (141, 165). It is now well established that cancer cells and other hyperproliferative cells reprogram their metabolism to predominantly perform glycolysis as opposed to mitochondrial respiration to generate metabolites required for their rapid growth (155). This metabolic switch is known as the Warburg effect and is an important hallmark of cancer cells (50). It has recently been established that colorectal cancer patients have a specific metabolic signature compared to healthy patients. Interestingly, this study established that there were distinct metabolic differences for colorectal tumours at different stages, corroborating the notion that tumour initiation and progression are processes that are the result of an abnormal usage of energy by cells (166). Within the last two decades, protein kinases as well as phosphorylation events have been observed in the mitochondria. This revelation led to the hypothesis that the mitochondrial machinery may be regulated by proteins that are normally cytosolic or localized to other organelles. Several mitochondrial complexes or proteins have been shown to be regulated by phosphorylation (143, 144, 167). For instance, the pyruvate dehydrogenase complex (PDC) was the first mitochondrial phosphoprotein identified and it serves as a link between glycolysis and mitochondrial respiration, the two major energy-producing pathways. The PDC is regulated by phosphorylation and it is essential to maintain cellular glucose homeostasis (144). As such, mitochondria have been said to be an “underappreciated site of signaling by phosphorylation” (168). Considering that receptor tyrosine kinases and phosphatases

regulate a fine homeostasis of phosphorylation events in cells that are essential for proper cellular processes and responses, the presence of RTKs and reversible phosphorylation in the mitochondria brought up the question of whether protein phosphatases could also be present in this organelle. Indeed, several PTPs have been established to be present in the mitochondria. The first PTP found to be localized in the mitochondria was PTPMT1, which was identified by its CX_5R PTP signature motif (169). It is localized to the mitochondria through its N-terminal motif which is recognized by a mitochondrial translocase and is anchored to the inner mitochondrial membrane (IMM) at the matrix face, where the ETC consisting of proton pumps and ATP synthase reside (169, 170). The PTPs SHP-2 and PTP1B have been found in brain mitochondria and appear to be guided there through their tail anchor (168, 171). DUSP18, DUSP21 and DUSP1 were found to be localized at the outer mitochondrial membrane (OMM), the latter of which localizes there following specific stimuli such as p38 MAPK activation (69, 70). One group has identified PTPD1 (gene name *PTPN21*) to be targeted to the OMM via AKAP-121, a PKA-anchoring protein (172). Interestingly, this AKAP-121/PTPD1/PKA signaling complex has been proposed to regulate ATP synthesis by modulating the metabolism of the mitochondria via the activation of cAMP and Src (173). Possessing the knowledge that some PTPs do localize to the mitochondria and that some regulate the metabolic reprogramming, we sought to determine whether other PTPs could regulate the Warburg effect by either promoting or dampening mitochondrial respiration and function.

In this study, I have demonstrated through the use of a human phosphatome metabolic screen that PTPs can indeed regulate colorectal cancer bioenergetics through modulation of mitochondrial respiration. More specifically, we determined that 24 PTPs

modulate the mitochondrial respiration of cancer cells: 16 positively regulate mitochondria-dependent respiration while 8 PTPs negatively regulate it. Of these candidates, TC-PTP emerged as a true regulator of colorectal cancer bioenergetics. With additional studies we validated its role in promoting mitochondrial respiration in colorectal cancer cells. While absence of TC-PTP led to a decrease in the respiration performed through the mitochondria, glycolysis was unaffected, indicating that TC-PTP could act as a negative regulator of the Warburg effect.

2.2 Materials and Methods

2.2.1 Cell culture

HEK 293T/17 (ATCC) and HCT116 colorectal cancer cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (FBS, Gibco) and 25 mg gentamycin (Wisent) in a 5% CO_2 incubator at 37°C. Stable cell lines HCT116 shFF, shPTPN2 (1), shPTPN2 (2), shPTPN2 (3) shPTPN2 (4) and shPTPN2 (Pool) were cultured in selection media (DMEM supplemented with 10% FBS, 25 mg gentamycin and 2 µg/mL puromycin) in a 5% CO_2 incubator at 37°C. For specific experiments, cells were stimulated with 10% FBS, 1.5 µM FCCP (Sigma-Aldrich), recombinant human IL-10 (PeproTech) or recombinant human IL-6 (PeproTech) for the defined amount of time.

2.2.2 Plasmid transfection and infection for phosphatome metabolic screen

HEK 293T/17 cells were plated in a 96-well plate at a density of 25,000 cells/well. 24 hours later, they were transfected with 100 ng of a pool of 4 to 6 shRNA specifically targeting each PTP gene using Lipofectamine 2000 (Sigma-Aldrich), according to the

manufacturer's protocol. Cells were incubated for 48 hours in a 5% CO_2 incubator at 37°C. To perform the metabolic Seahorse assays, HCT116 cells were plated directly in the flux pack 96-well microplate at a density of 7,500 cells/well and incubated in a 5% CO_2 incubator at 37°C. Viral supernatant from HEK 293T/17 cells was retrieved and used to infect the HCT116 cells 24 hours after seeding. 48 hours after infection, the metabolic Seahorse assay was performed using the shRNA-infected HCT116 cells.

2.2.3 Generation of stable cell lines

Stable cell lines targeting *PTPN2* were generated by transfecting HEK 293T/17 cells with the individual shRNAs against *PTPN2* (1, 2, 3 and 4) as well as the four combined shRNAs (1 to 4, Pool) in a 6-well plate and incubating them in a 5% CO_2 incubator at 37°C. 24 hours post-transfection, HCT116 cells were plated in a 6-well plate and similarly incubated. 24 hours after seeding, HCT116 cells were infected with viral supernatant collected from HEK 293T/17 cells and filtered using a 0.45 µm filter. 48 hours after infection, HCT116 cells were washed and media was replaced with fresh selection media and incubated in a 5% CO_2 incubator at 37°C. When cells were confluent, they were transferred to a 10 cm^2 petri dish and maintained in selection media for 2 weeks to select for the infected cells. Cells were passed when confluent and maintained in DMEM supplemented with 10% FBS and 25 mg gentamycin after the selection period.

2.2.4 Extracellular Flux Analyzer

Metabolic changes of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using an XF96 Extracellular Flux Analyzer (Agilent) based on the level of oxygen and on the number of protons found in the extracellular media, respectively. For the metabolic screen, HCT116 cells were infected directly in the XF96

microplates as mentioned previously, ready for the assay. For the stable cell lines, 30,000 cells per well were seeded in 80 µL media in a XF96 microplate and incubated in a 5% CO_2 incubator at 37°C for 24 hours. On the day of the assay, 50 µL of the media was gently removed from the wells using a multichannel pipette, cells were washed twice with XF assay medium (Agilent) and fresh XF assay medium was added. The plate was incubated for 1 hour in a CO_2-free incubator at 37°C for the equilibration period. The cell plate was then transferred to the XF96 extracellular flux analyzer and the assay performed, where one cycle comprises of a 2-minute mix, 2-minute wait and 3-minute measure period. After 4 basal assay cycles, 4 cycles after each drug injection were performed. To measure mitochondrial respiration, oligomycin (Sigma-Aldrich), FCCP and rotenone (Sigma-Aldrich) were added at specific time points to inhibit different complexes of the electron transport chain (ETC) at a final concentration of 1.5 µM, 0.5 µM and 1 µM, respectively. For the glycolytic assays, glucose (Fischer Scientific), oligomycin and 2-deoxy-D-glucose (2-DG; Sigma-Aldrich) were injected at a final concentration of 10 mM, 1.5 µM and 100 mM, respectively. Each assay was performed with at least 5 replicates and normalized to cell number by performing a CyQUANT assay, as per manufacturer's recommendations.

2.2.5 Intracellular ATP assay

25,000 cells/well of HCT116 cells were plated in a 96-well plate and incubated in a 5% CO_2 incubator at 37°C for 48 hours. Cells were then stimulated or not with 1.5 µM FCCP for 24 hours. Media was removed from the wells and the cells were frozen at -80°C. A CellTiter-Glo Luminescent Buffer and Substrate assay was performed, according to manufacturer's recommendations. Briefly, the CellTiter-Glo Buffer vial was

transferred to the Substrate vial and mixed well by vortexing. 100 µL of this mixture was added per well and let to incubate at room temperature on a plate shaker for 2 minutes. Contents were transferred to a white 96-well plate and further incubated at room temperature for 10 minutes, protected from light. Luminescence at 480 and 520 nm was recorded using a SpectraMax i3 and values were normalized to cell number as measured by a CyQUANT assay.

2.2.6 Proliferation assays

7,500 cells/well of HCT116 cells were plated in a 96-well plate and placed in the IncuCyte ZOOM system (37°C and 5% CO_2) for 110 hours with or without 1.5 µM FCCP. Media was changed for all wells every 48 hours, and fresh FCCP added to the appropriate wells. Cell proliferation was recorded in real-time, and the coverage of each well (percent confluency) was assessed and plotted.

2.2.7 Western blotting

HCT116 cells were stimulated or not with 1.5 µM FCCP, 10% FBS, 100 ng/mL IL-10 and 10 ng/mL IL-6 in a 6-well plate or 10-cm^2 petri dish, and at the specified time points, cells were washed twice with PBS and frozen at -80°C. Total cell lysates (TCLs) were then prepared using modified radioimmunoprecipitation assay (mRIPA) buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.25% sodium deoxycholate and 1% NP-40) supplemented with ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor (Roche) and 2 mM sodium orthovanadate (Sigma-Aldrich). Protein levels were quantified by bicinchoninic acid (BCA) assay. 20 µg of each TCL was ran on 10% SDS-PAGE gels using the following anti-mouse primary antibody: TC-PTP (1/10, homemade clone 3E2), as well as the following anti-rabbit primary antibodies from Cell Signaling Technology:

STAT3 (1:1,000), p-STAT3 (1:1,000, Tyr705), STAT1 (1:1,000), p-STAT1 (1:1,000, Tyr701), p38 (1:1,000), p-p38 (1:1,000, Thr180/Tyr182), NF-κB (1:1,000), p-NF-κB (1:1,000, Ser536), ACLY (1:1,000) and p-ACLY (1:1,000, Ser454). The anti-rabbit primary antibody calnexin (1:50,000) was provided from J.J.M. Bergeron (McGill University), and secondary antibodies anti-rabbit and anti-mouse horseradish peroxidase (HRP, 1:10,000) were obtained from Jackson ImmunoResearch. Blots were imaged using the Mini-Medical Series imager (AFP Imaging).

2.2.8 RNA isolation, reverse transcription and quantitative real-time PCR

Total ribonucleic acid (RNA) was isolated from HCT116 cells using the TRIzol reagent (Thermo Fisher Scientific), according to the manufacturer's protocol. RNA was quantified with NanoDrop and the quality of RNA was determined by running an RNA gel using 500 ng to 1 µg RNA on a 1% agarose gel. 1 µg of RNA was reverse-transcribed to cDNA using the SuperScript III Reverse Transcriptase Kit (Thermo Fisher Scientific), according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with a LightCycler 480 (Roche) using SYBR Green Master Mix (Roche), according to the manufacturer's recommendations. A panel of different housekeeping genes was tested with these cell lines. *ACTB* was used as a reference gene to normalize the relative abundance of each gene of interest since it was determined to be the most stable reference gene in our particular cell lines and experimental setup.

2.2.9 Statistical analysis

All but one experiment conducted were analyzed using one-way analysis of variance (ANOVA) with a Dunnett post-test. The intracellular ATP assays were analyzed using two-way analysis of variance (2way ANOVA) with a Tukey post-test.

2.3 Results

2.3.1 A subset of PTPs regulate the metabolism of colorectal cancer cells

To date, only a handful of PTPs have been shown to regulate the Warburg effect. PTPMT1 (174) and SHP-1 (175) have been shown to be negative regulators of the Warburg effect. Knowing the mitochondrial localization of several PTPs as well as the role of some members of the family in regulating the Warburg effect, we sought to determine whether other PTPs could regulate the metabolic reprogramming of cancer cells by either promoting or dampening mitochondrial respiration and function. Considering that colorectal cancer is the third most frequent type of cancer worldwide and knowing that metabolic alterations are frequent in CRC, we chose it as our model for the human metabolic screen. We first had to design a method in which hundreds of PTPs could be analyzed for their potential role in regulating cancer bioenergetics. To do so, we used a PTP shRNA library and combined 3 to 6 shRNAs targeting each member of the PTP family to infect the colorectal cancer cell line HCT116 using virus produced from HEK 293T/17 cells directly in the Seahorse microplates and incubated the cells for 48 hours. Thereafter, the Seahorse extracellular flux assay was performed and readout of the mitochondrial respiration was obtained (**Figure 2.1**).

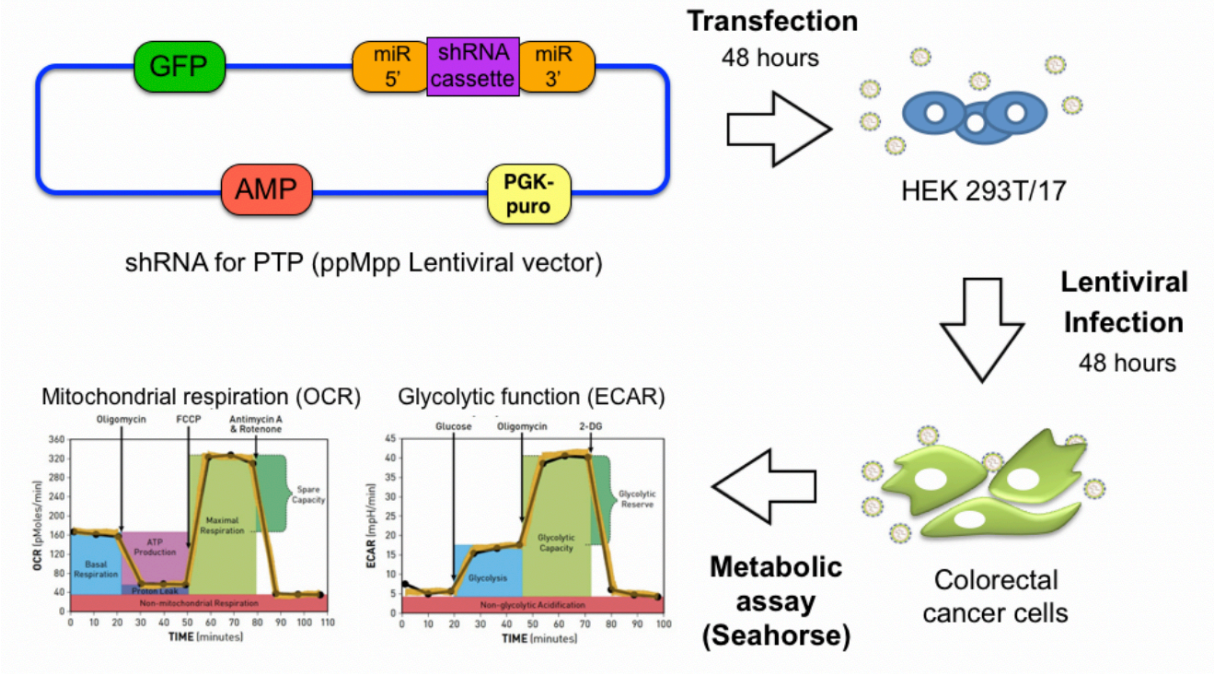

Figure 2.1: Methodology for metabolic screen. HEK 293T/17 cells were transfected with 3-6 shRNAs directed against a specific PTP for 48 hours. Viral supernatant was collected and used to infect HCT116 cells directly in the Seahorse microplate. 48 hours post-infection, the Seahorse assay was performed. The mitochondrial respiratory phenotype was determined based on the oxygen consumption rate (OCR) and the glycolytic activity was measured by the extracellular acidification rate (ECAR) of the cell media.

Out of the 72 individual PTP members successfully tested, 24 were found to play a role in regulating the mitochondrial respiration of colorectal cancer cells (**Figure 2.2**). We determined this by measuring the spare respiratory capacity (SRC) of the cells following inhibition of a given PTP, which represents the maximum respiration that a cell can undergo. This mimics a stress to the cell, such as hypoxia, nutrient deprivation or drastic changes in temperature. We found that inhibition of 16 of these PTP candidates downregulated the SRC of these cells, while inhibition of 8 PTPs promoted cellular mitochondrial respiration (**Figure 2.2**), indicating a considerable participation of diverse PTPs in regulating the metabolism of colorectal cancer cells.

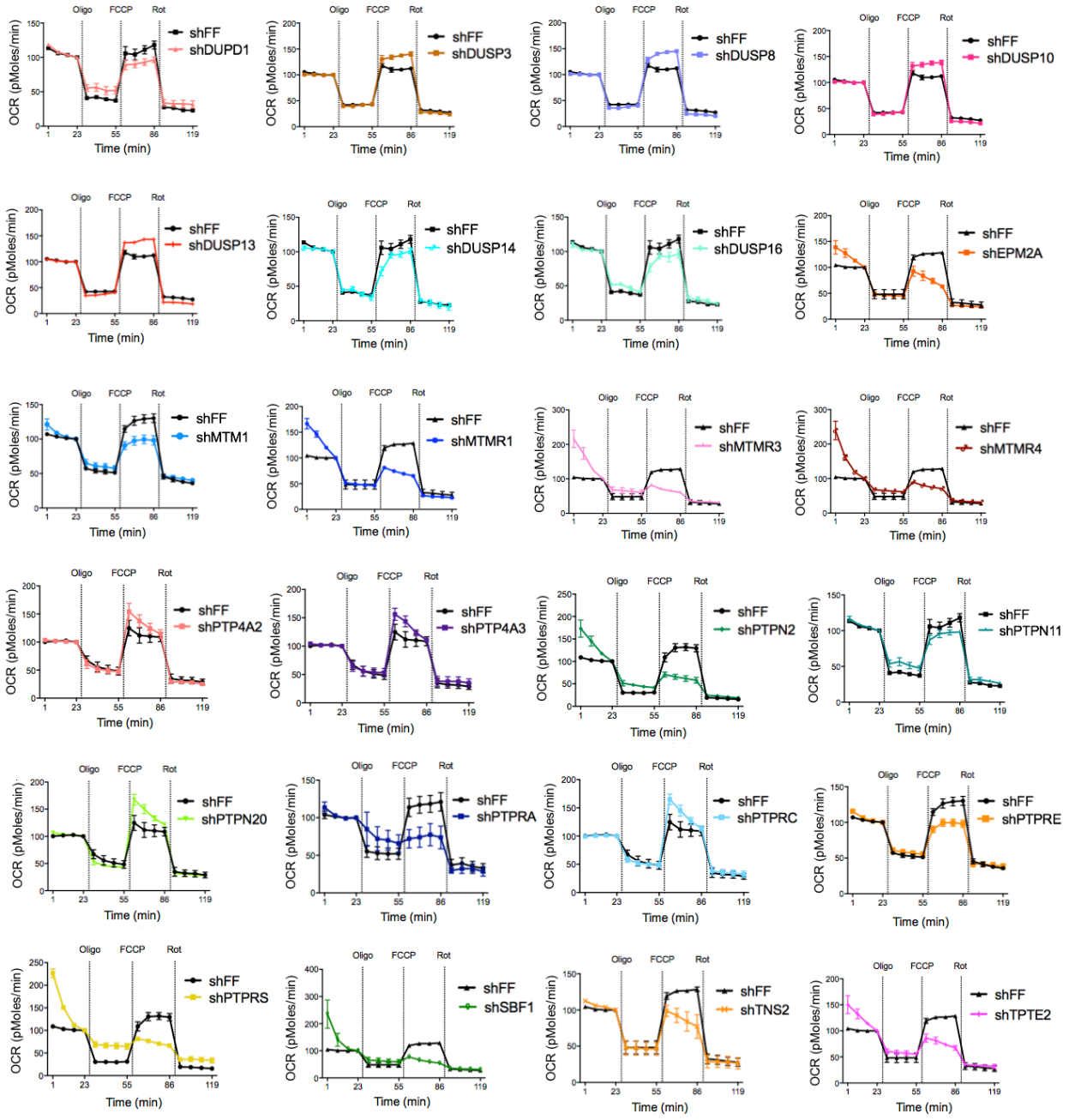

Figure 2.2: A human phosphatome metabolic screen identifies 24 protein tyrosine phosphatases as candidates for regulating the metabolism of colorectal cancer cells. A large shRNA PTP library consisting of about 500 shRNAs was used to infect colorectal cancer cells HCT116 to study whether their metabolism would be affected upon inhibition of a specific PTP. Representative mitochondrial respiratory profiles of the 24 PTP candidates that were found to regulate the spare respiratory capacity (SRC) of these cells, either positively (16) or negatively (8). Experiments performed with at least 5 replicates.

2.3.2 Absence of TC-PTP leads to inhibition of mitochondrial respiration

After completing the metabolic screen, we noticed that inhibition of two PTPs led to a very drastic decrease in mitochondrial respiration: TC-PTP and PTPRS. Considering that two substrates of TC-PTP, STAT1 and STAT3, have been shown to localize to the mitochondria, we decided to further study the involvement of TC-PTP (gene name *PTPN2*) in regulating the metabolism and downstream signaling pathways of colorectal cancer cells. We first generated stable cell lines with the individual shRNAs targeting *PTPN2* (1 to 4) as well as a pool of the 4 shRNAs combined and confirmed efficient knockdown of TC-PTP by western blot for protein levels (**Figure 2.3A**) and qRT-PCR for gene expression (**Figure 2.3B**). We selected shRNA #2 and 3 for additional studies as they demonstrated the best knockdown of TC-PTP. To confirm the results obtained from the metabolic screen with the four pooled shRNAs, we repeated the Seahorse assays with the stable cell lines and obtained similar results. Inhibition of TC-PTP using individual shRNAs resulted in a decrease in the mitochondrial respiration of colorectal cancer cells (**Figure 2.3C**), as demonstrated from the reduction of the OCR upon injection with the membrane uncoupler FCCP, which represents the SRC (**Figure 2.3D**). These experiments also highlighted the fact that knockdown of TC-PTP in these cells did not cause changes in the basal respiration (**Figure 2.3E**), ATP production (**Figure 2.3F**) or uncoupled respiration (**Figure 2.3H**). The specific decrease in SRC in TC-PTP-deficient cells indicated that these cells would be unable to adapt to stress, such as is the case during cancer progression with nutrient deprivation or hypoxia (176-179).

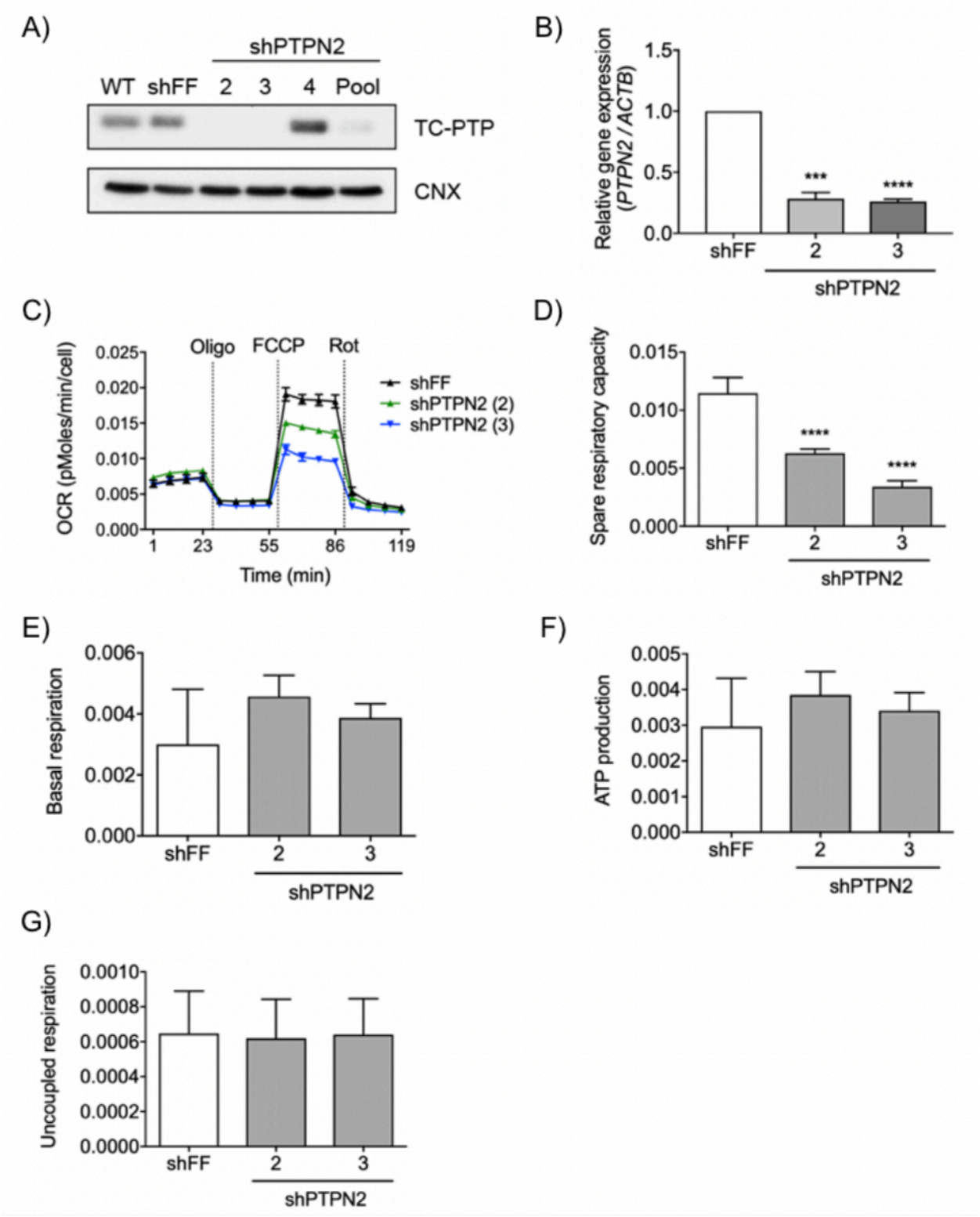
A)
shPTPN2
WT shFF 2 3 4 Pool
TC-PTP
CNX
B)
Relative gene expression (PTPN2 / ACTB)
1.5
1.0
0.5
0.0
***
****
shFF 2 3
shPTPN2
C)
Oligo FCCP Rot
OCR (pMoles/min/cell)
0.025
0.020
0.015
0.010
0.005
0.000
shFF
shPTPN2 (2)
shPTPN2 (3)
1 23 55 86 119
Time (min)
D)
Spare respiratory capacity
0.015
0.010
0.005
0.000
****
****
shFF 2 3
shPTPN2
E)
Basal respiration
0.006
0.004
0.002
0.000
shFF 2 3
shPTPN2
F)
ATP production
0.005
0.004
0.003
0.002
0.001
0.000
shFF 2 3
shPTPN2
G)
Uncoupled respiration
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
shFF 2 3
shPTPN2

Figure 2.3: TC-PTP-deficient cells have a decreased mitochondrial respiration upon induction of stress. Human colon cancer cells HCT116 were infected with 4 shRNA plasmid constructs targeting *PTPN2* and selected for two weeks with puromycin to generate stable cell lines (HCT116 shPTPN2 #2, #3, #4 and Pool). (A) Total cell lysates were used to perform western blot to confirm efficient knockdown of TC-PTP and was quantified by densitometry. (B) qRT-PCR was done to analyze the gene expression of *PTPN2*, the gene encoding for TC-PTP, which was significantly lower in the shPTPN2 stable cells lines. (C-G) The oxygen consumption rate (OCR) of control (shFF) and HCT116 shPTPN2 cells was measured to establish their mitochondrial respiratory profile. HCT116 shPTPN2 cells had a decreased mitochondrial respiration (C) and spare respiratory capacity (D) compared to shFF cells in black. On the other hand, basal respiration (E), ATP production (F) and uncoupled respiration (G) were unchanged in these cells. Values are normalized to cell number. Data consist of mean ± SD and are representative of four independent experiments of 5 to 10 replicates. Statistical analysis performed by one-way ANOVA with multiple comparisons test. ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$. Oligo: oligomycin; FCCP: carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone; Rot: rotenone.

2.3.3 Glycolysis is unaffected in absence of TC-PTP

In cancer cells, the Warburg effect reflects a metabolic switch whereby cancer cells will preferentially undergo glycolysis as opposed to mitochondrial respiration, even in the ample presence of oxygen. Since an eminent hallmark of cancer is this reprogramming of metabolism, we wondered if TC-PTP-deficient cells having a much lower mitochondrial respiration would overcome this by reprogramming their metabolic pathways to promote glycolysis. To do so, we performed a modified version of the Seahorse assay conducted for the metabolic screen by performing a glycolytic assay with the HCT116 stable cell lines. We found that inhibition of TC-PTP in HCT116 cells did not modulate glycolysis, as demonstrated by the extracellular acidification rate (ECAR) profile (**Figure 2.4A**), glycolytic capacity (**Figure 2.4B**), glycolytic reserve (**Figure 2.4C**) and glycolysis **(Figure 2.4D**). It appears that the role of TC-PTP in these colorectal cancer cells is mostly to upregulate their utilization of mitochondria, thus favouring mitochondrial respiration and reprogramming their energy usage. In this sense, it would seem that TC-PTP may act as

a negative regulator of the Warburg effect, increasing the cellular requirement for energy generation through oxidative phosphorylation.

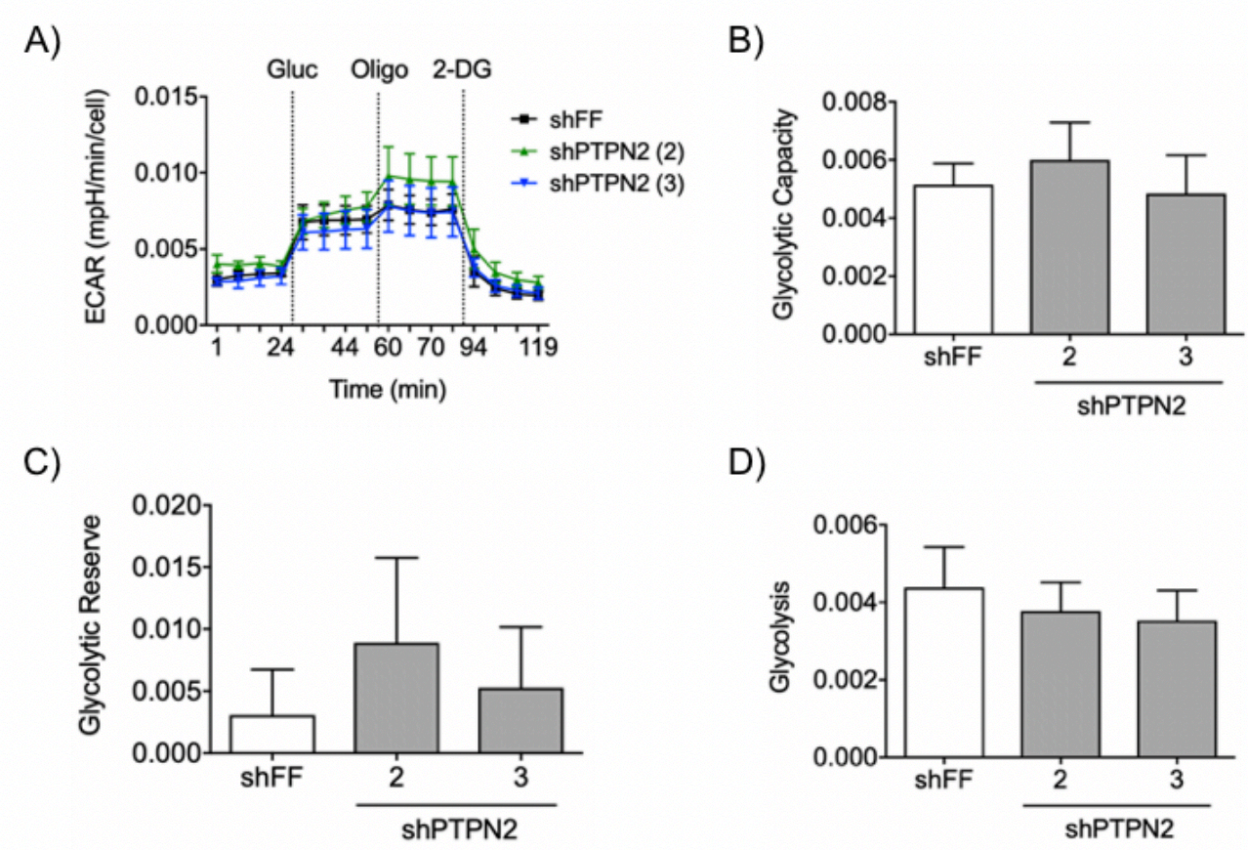

Figure 2.4: Glycolytic activity is unchanged in colorectal cancer cells lacking TC-PTP. The glycolytic activity of HCT116 shPTPN2 stable cell lines was measured using the Seahorse XF96 analyzer to measure extracellular acidification rate (ECAR) under basal condition and in response to glucose (Gluc, 10 mM), oligomycin (Oligo, 1.5 µM) and 2-Deoxy-D-glucose (2-DG, 100 mM). (A) ECAR is not affected in TC-PTP-deficient cells (shPTPN2) compared to control cells (shFF). The glycolytic capacity (B), glycolytic reserve (C) and glycolysis (D) are similarly unaffected in these cells. Values are normalized to cell number. Data consist of mean ± SD and are representative of five independent experiments with 5 to 10 replicates. Statistical analysis was performed by one-way ANOVA with multiple comparisons test.

2.3.4 Ablation of TC-PTP results in decreased levels of intracellular ATP and proliferation specifically upon induction of stress

Given that we observed an important reduction of mitochondrial respiration in TC-PTP-deficient cells, we wanted to delve deeper into other properties that are characteristic of cancer cells. More specifically, since we observed a reduction in the mitochondrial respiration in these cells, we were interested in determining whether ATP synthesis would be affected. We know that mitochondria are the famous “powerhouse of the cell” and that they are the main source of ATP production (141), so if the cells were employing this energy factory less, they may also be producing less ATP as well. To confirm our hypothesis, we measured the intracellular levels of ATP in control and TC-PTP-deficient cells under basal conditions and upon induction of stress with FCCP. We found that at the basal state, there was no difference in ATP production between cells. However, in response to stress induced by stimulation with FCCP, there was a general reduction in intracellular ATP levels for all cells, whether TC-PTP was present or not, but this decrease was further enhanced in absence of TC-PTP (**Figure 2.5A**). Given that the drugs used in the Seahorse assay inhibit different complexes of the ETC and that ATP synthesis is impaired upon inhibition of TC-PTP, these assays emphasize the role of TC-PTP in positively modulating the mitochondrial machinery and ATP synthesis in conditions of stress. Another hallmark of cancer is sustained proliferative signaling (50). After observing a decrease in intracellular ATP in TC-PTP-deficient cells to accompany their lower usage of the mitochondria, we performed various proliferation assays to determine whether these cells had a deficiency in their proliferative potential. We performed IncuCyte proliferation assays under basal conditions and upon induction of stress with FCCP. We observed that cell proliferation was unaffected by the presence or

absence of TC-PTP under basal conditions (**Figure 2.5B**). Nonetheless, all cells stimulated with FCCP had a lower rate of proliferation than their respective unstimulated controls, but it is still unclear whether loss of TC-PTP further decreased the rate of proliferation. A small shift in cell confluency was observed in the exponential growth phase of TC-PTP-deficient cells, meaning that there might be a delay in growth. Absence of TC-PTP may cause a reduction in proliferation of colorectal cancer cells, but more assays would need to be performed to confirm (**Figure 2.5B**).

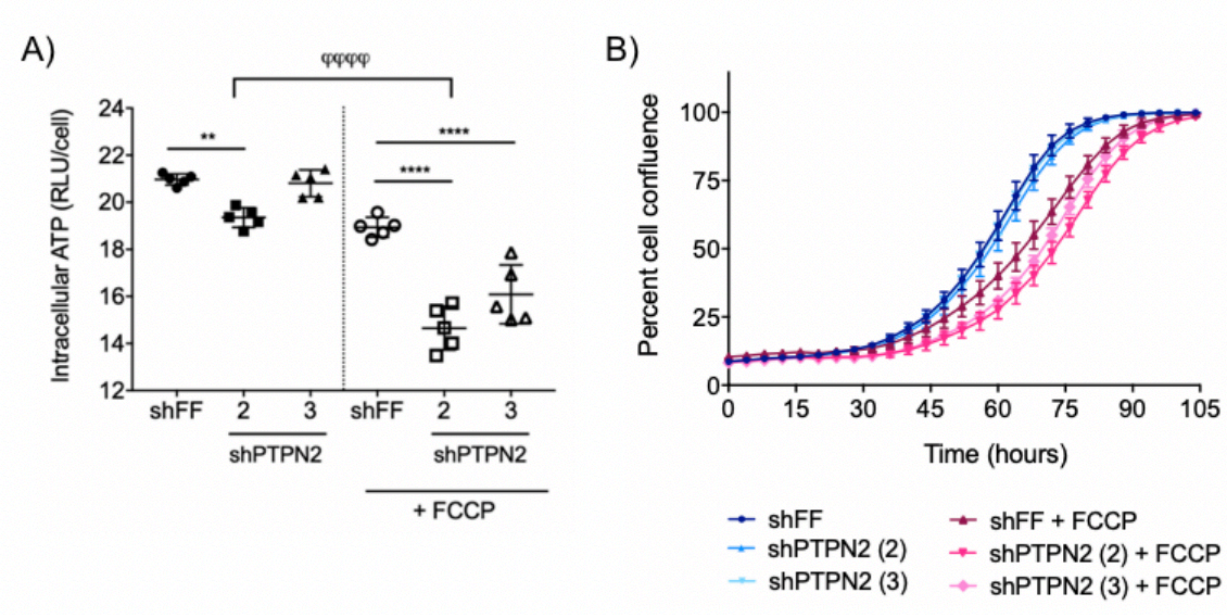

Figure 2.5: Decreased intracellular ATP levels and proliferation in colorectal cancer cells upon induction of stress. (A) Intracellular ATP levels were measured in HCT116 cells under basal conditions and after stimulation with 1.5 μM FCCP for 24 hours. At the basal state, there was no difference in ATP levels between control cells (shFF) and TC-PTP-deficient cells (shPTPN2). Stimulation with FCCP resulted in a general reduction in intracellular ATP levels which was further enhanced in absence of TC-PTP. Samples are normalized to cell number. Data consist of mean ± SD and are representative of three independent experiments performed with five replicates. *** $p < 0.05$, *** $p < 0.001$, φφφφ $p < 0.001$ between FCCP-stimulated cells and respective unstimulated samples. (B) 7,500 cells/well were plated in a 96-well plate and an IncuCyte proliferation assay was performed for a period of 110 hours. FCCP was added to the appropriate wells every 48 hours. Slopes indicate the percent cell confluency in real time, which represents cell proliferation. Data are representative of three independent experiments performed with six replicates.

2.3.5 TC-PTP inhibition leads to hyperphosphorylation of STAT1, STAT3 and p38

To better understand how inhibition of TC-PTP in colorectal cancer cells could lead to such a reduction in mitochondrial usage and ATP synthesis, we analyzed the protein abundance of known substrates of TC-PTP and other proteins involved in cancer cell survival and metabolism, notably STAT1, STAT3, p38 MAPK and ACLY, among others. STAT1 and STAT3 are known substrates of TC-PTP (89, 90), and have recently been demonstrated to be present in mitochondria (150, 151, 153, 180). In addition, STAT3 seems to directly regulate the ETC through regulation of complexes I, II and V (181). STAT1, p38 and NF-κB induce the expression of genes involved in cell survival, while ACLY is an enzyme that regulates an important step of fatty acid biosynthesis. By western blotting, we demonstrated that inhibition of TC-PTP resulted in hyperphosphorylation of STAT1 and STAT3, which are both involved in JAK/STAT signaling pathways and can be activated by interferons, IL-10 or IL-6. We also observed upregulation of p38 phosphorylation in absence of TC-PTP and observed no difference in the phosphorylation levels of NF-κB or ACLY between cells with or without TC-PTP (**Figure 2.6**). We are repeating these studies with IL-6 and IL-10 stimulations to determine whether expression of STAT1, STAT3 and p38 will be further upregulated upon activation of these JAK/STAT pathways. The hyperphosphorylation of STAT1, STAT3 and p38 indicate that these signaling pathways are upregulated in absence of TC-PTP. Whether their hyperactivation is a consequence or a cause of the altered mitochondrial respiration has not yet been established but would be very important to investigate.

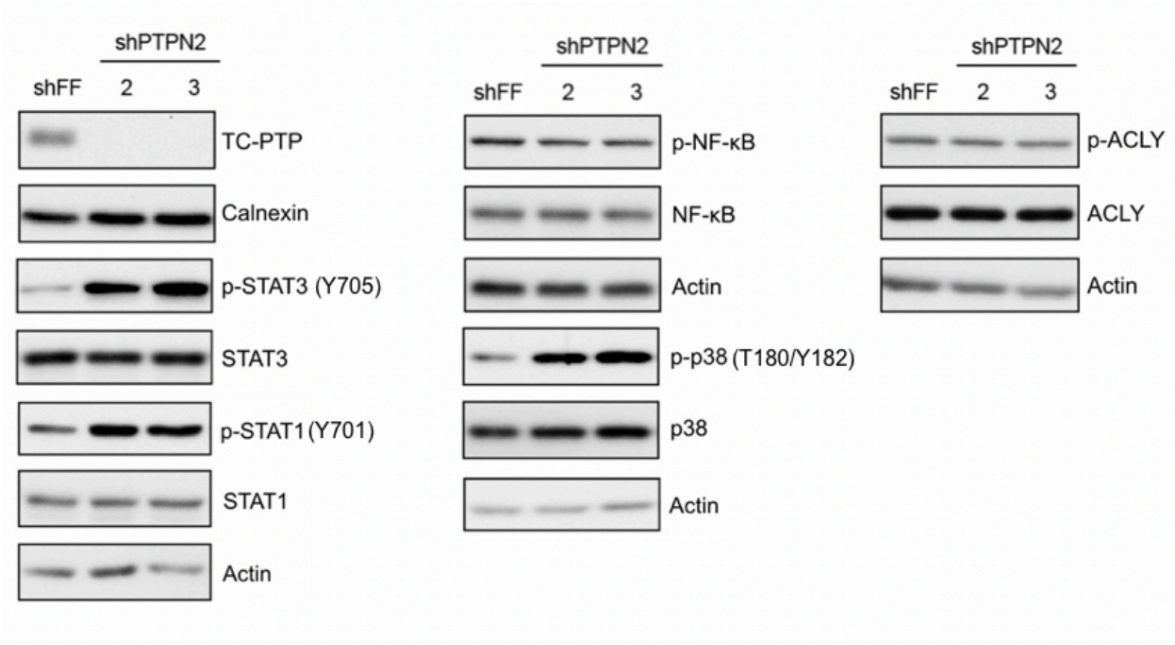

Figure 2.6: Hyperphosphorylation of STAT3, STAT1 and p38 in absence of TC-PTP. Total cell lysates from HCT116 shFF and shPTPN2 stable cell lines were used to perform western blotting against known substrates of TC-PTP (STAT3 and STAT1) as well as proteins involved in cancer cell survival, proliferation and metabolism (NF-κB, p38 and ACLY). Absence of TC-PTP resulted in hyperphosphorylation of STA3, STAT1 and p38, while NF-κB and ACLY phosphorylation seemed to be unaffected. Blots are representative of four to five independent experiments.

2.3.6 Metabolic reprogramming in TC-PTP-deficient cells is not due to aberrant transcription of metabolic genes

Having shown that loss of TC-PTP results in the upregulation of several signaling pathways, we next aimed to determine whether these changes could also be observed at the transcriptional level, particularly regarding the expression of genes involved in different metabolic pathways. The panel of metabolic genes that we established comprised genes involved in regulating components of the electron transport train and OXPHOS (*ATP5J* and *NDUFB5*), members that regulate rate-limiting steps of glycolysis (*HK2*, *PFKL*, *PFKM* and *PFKP*), enzymes of fatty acid biosynthesis (*ACACA* and *ACLY*) and finally those encoding enzymes that reduce oxidative stress (*CAT* and *SOD1*). We

performed qRT-PCR with colorectal cancer cells under normal conditions or upon induction of stress by treating cells with 1.5 µM FCCP for 1 to 24 hours. In basal state, we observed no differences in the expression of these metabolic genes in absence or presence of TC-PTP (**Figure 2.7**). However, preliminary data indicate that this may not be the case when cells are in a stressed environment. After uncoupling the flow of electrons from ATP production with FCCP for 18 hours, we observed an increase in several metabolic genes in absence of TC-PTP and a decrease in some of their expression at 24 hours, notably of *ACACA*, *ATP5J*, *CAT*, *HK2*, *NDUFB5* and *PFKL* (**Figure 2.7**). This could indicate that TC-PTP regulates the expression of distinct metabolic genes in a time-dependent fashion, but this would need to be validated and further studied. Similar results were obtained under nutrient-deprivation conditions. Perhaps these data could suggest that loss of TC-PTP in colorectal cancer cells is not sufficient to alter their metabolic transcriptome under basal conditions, but coupled to a cellular stress such as FCCP stimulation may lead to a reprogramming of genes involved in regulating their metabolism.

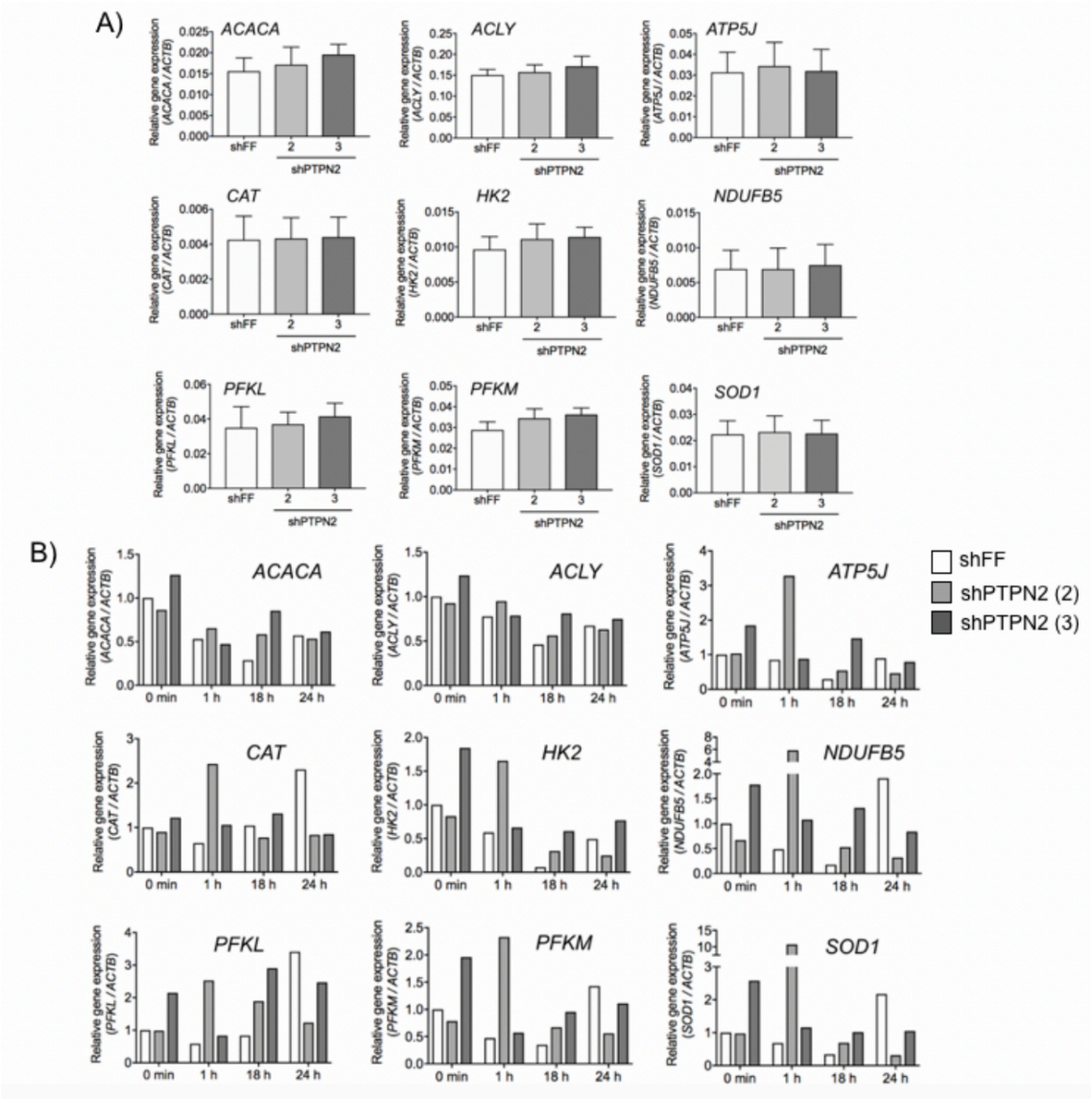

Figure 2.7: Altered metabolism in TC-PTP-deficient colorectal cancer cells is not due to aberrant transcription of metabolic genes. RNA was isolated from HCT116 stable cell lines and qRT-PCR was performed to determine the gene expression of several genes involved in regulating OXPHOS (*ATP5J*, *NDUFB5*), glycolysis (*HK2*, *PFK*s), fatty acid synthesis (*ACACA*, *ACLY*) or diminishing oxidative stress (*CAT*, *SOD1*). At basal state, there were no differences in the expression of any of the genes analyzed between cells with or without TC-PTP. Data represents mean ± SD of four independent experiments performed in triplicate. (B) Cells were treated with 1.5 μM FCCP for 1 to 24 hours to induce stress and qRT-PCR was performed with the same metabolic genes. There appears to be an upregulation of the expression of several metabolic genes after 18 hours of induction of stress when TC-PTP is inhibited. Data represent mean ± SD representative of one or two independent experiments performed in triplicate. All samples were normalized to the reference gene *ACTB*.

Of the genes analyzed (**Table 2.1**), *ACACA*, *ACLY*, *ATP5J*, *HK2*, *NDUFB5*, *PFKL* and *PFKM* seemed to be upregulated after 18 hours of stimulation with the membrane uncoupler FCCP. However, the relative gene expression of *CAT*, *NDUFB5*, *PFKL*, *PFKM* and SOD1 appeared to be decreased after 24 hours of stimulation. It will be interesting to elucidate whether there is a time-dependent expression of *NDUFB5*, *PFKL* and *PFKM* when TC-PTP is not there to regulate various survival and metabolic pathways.

Table 2.1: List of human primers used for qRT-PCR

Metabolic gene	Forward primer (5' - 3')	Reverse primer (5' - 3')
ACACA	TCGTTGTCATGGTCACACCT	TGTTGTTGTTTGGTCCTCCA
ACLY	TGCCGACTACATCTGCAAAG	GGTTCAGCAAGGTCAGCTTC
ACTB	CTCTTCCAGCCTTCCTTCCT	AGCACTGTGTTGGCGTACAG
ATP5J	CTGGAGGACCTGTTGATGCT	TGGGGTTTTTCGATGACTTC
CAT	CGTGCTGAATGAGGAACAGA	AGTCAGGGTGGACCTCAGTG
GAPDH	GCCGCATCTTCTTTTGCGT	CCAATACGACCAAATCCGTTGA
HK2	AGTGGAGTGGAAGGCAGAGA	TCATTCACCACAGCCACAAT
NDUFB5	CCAGAAGGCTATGTCCCAGA	CGCACTTCCAGCTCCTTTAC
PFKL	ATGTGGGTGCCAAAGTCTTC	CAGCTGGATGATGTTGGAGA
PFKM	AGAGCGTTTCGATGATGCTT	GTTGTAGGCAGCTCGGAGTC
PTPN2	GACAGGTGACCGATGTACAGG	CTGCACCTTCTGAGCTGTGGT
SOD1	GAAGGTGTGGGGAAGCATTA	ACATTGCCCAAGTCTCCAAC

2.3.7 Human phosphatome metabolic screen identifies 24 PTP candidates that regulate colorectal cancer bioenergetics

By performing additional experiments regarding the effects of TC-PTP inhibition on colorectal cancer cell metabolism and seeing that these confirmed those obtained from the screen, we are confident that the human phosphatome metabolic screen is a useful

tool that can shed light on the PTPs that can be involved, either directly or indirectly, in regulating the metabolism of cancer cells. Of note, of the PTPs tested, there were twice as many that could act as positive regulators of mitochondrial function compared to negative regulators (**Figure 2.8**). This metabolic screen could be a very useful tool to identify targets that control metabolic reprogramming in cancer cells. It will be very interesting to corroborate other candidates in our model study, and even to expand this screen in other systems and cancer types.

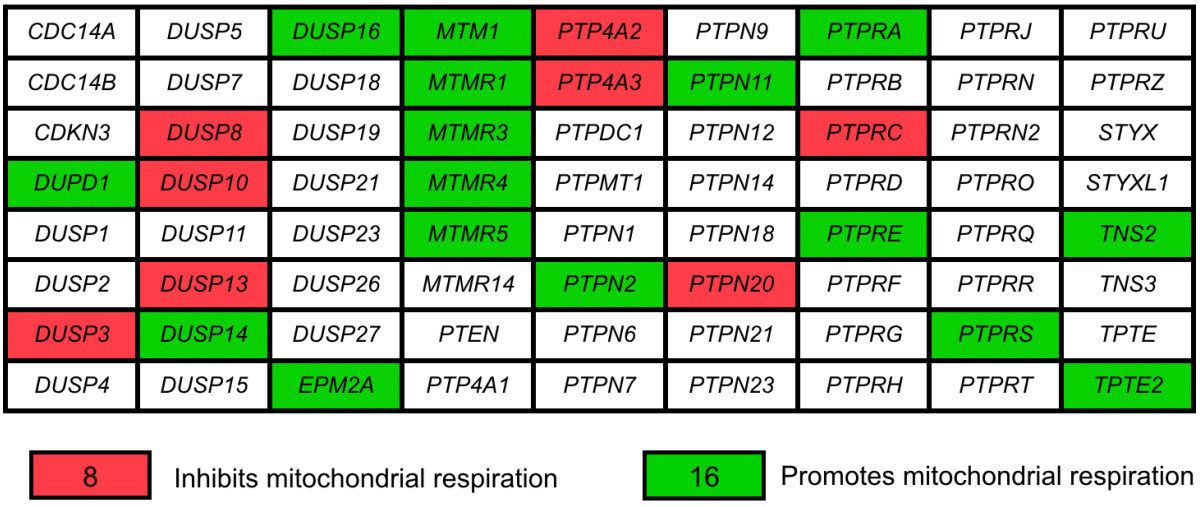

Figure 2.8: Summary of human phosphatome metabolic screen. After performing Seahorse metabolic assays with shRNA targeting each member of the PTP superfamily and analyzing their mitochondrial respiration and spare respiratory capacity, we identified 24 PTP candidates. We observed that 8 PTPs negatively modulate mitochondrial respiration while 16 PTPs positively modulate it. *PTPN2*, or TC-PTP, was confirmed as a true positive regulator of mitochondrial respiration in colorectal cancer cells.

2.4 Discussion

Over the decades, we have come to understand that certain cellular processes are so aberrant and frequent in cancer cells that they become characteristic of the changes that cancer cells undergo not only to grow and survive, but to thrive. Dysregulating their

cellular metabolism to preferentially undergo aerobic glycolysis is one such hallmark of cancer cells. Nonetheless, there is much more to discover regarding the mechanisms that they use to promote this metabolic switch. Considering the large number of phosphoproteins and receptor tyrosine kinases present in the mitochondria, we postulated that protein tyrosine phosphatases may similarly have a role on mitochondrial proteins and as such promote the reprogramming of cancer cell metabolism.

In our study, we employed a large shRNA PTP library and performed a human phosphatome metabolic screen to identify PTPs that may be involved in regulating the metabolism of colorectal cancer cells. We identified 24 PTPs that modulate their mitochondrial respiration: 16 of which promote mitochondrial respiration while 8 inhibit mitochondrial respiration. Of these candidates, we further validated the role of TC-PTP in enabling metabolic adaptation. When inhibited in human CRC cells, TC-PTP deficiency resulted in a considerable decrease in mitochondrial respiration and in their maximal respiration performed under conditions of stress. Such a stress would arise in a cancer setting such as generated by hypoxia or nutrient deprivation in the tumour, increased oxidative stress or dysregulated cytokine signaling leading to immune cell infiltration (50). In these instances, cells lacking TC-PTP would be unable to overcome the stress as their ability to respire properly by using their mitochondria effectively seems diminished. It would be intriguing to further investigate other stressors that are often associated with cancer using TC-PTP-deficient cells. Induction of hypoxia or removal of nutrient sources should be sufficient to induce stress, and it would be interesting to observe whether these cells have a lower mitochondrial respiration in these conditions *in vitro*.

To study the effects of downregulated respiration through the mitochondria, we assessed the generation of ATP in CRC cells and observed a drastic decrease in ATP levels in TC-PTP-deficient cells upon induction of stress with the membrane uncoupler FCCP. This emphasizes that mitochondria and most likely the ETC are dysfunctional and not generating the required amount of energy for the cell (50). The proliferation of TC-PTP-deficient cells also seems to be delayed upon induction of stress, but this would have to be further validated. However, we have previously demonstrated that TC-PTP-deficient IECs have an increased proliferation, specifically the colonic stem cells, indicating a role of TC-PTP in regulating proliferation (182). It would also be important to determine if cell migration is similarly affected when there are two stressors such as absence of TC-PTP and stimulation with FCCP.

Considering that we did not observe an upregulation in aerobic glycolysis, it is probable that TC-PTP-deficient cells may have an increased demand for ATP, not for metabolites. Could these cancer cells be trying to merely survive as opposed to growing and proliferating rapidly? This begs the question of how detrimental absence of TC-PTP is in these cells. It would be interesting to study the effect of TC-PTP deficiency in colorectal cancer cells in presence of other cellular stresses that may promote a similar phenotype, such as hypoxia or nutrient deprivation, notably glucose and glutamine. Accumulation of oxidative damage and structural integrity of mitochondria should be assessed to explore this question. In this study, we did not investigate whether TC-PTP alters glutamine metabolism. Since glutamine can be used depending on the context to contribute to the flux of carbons to the TCA cycle through glutaminolysis, cells that are dependent on glutamine for their metabolism may favour oxidative respiration through

upregulation of the TCA, thus being counter to the Warburg effect. The main steps of glutaminolysis involve the de-amination of glutamine to glutamate, followed ultimately by the conversion of glutamate to α-ketoglutarate, which can be accompanied or not by generation of alanine, aspartate or phosphoserine, depending on the enzymes involved (183). The entry of α-ketoglutarate in the TCA cycle results in the net gain of carbons in the cycle and thus increased activity. Some cancers, such as KRAS-driven cancers, rely more on glutamine metabolism to maintain their TCA cycle and support biosynthesis (184, 185). HCT116 colorectal cancer cells possess the KRAS mutation G13D which is thought to be a good prognostic factor of successful chemotherapy using cetuximab (186, 187), so it is conceivable that these cells are metabolically flexible and can depend on glutamine rather than glucose for respiration. As such, determining whether TC-PTP alters glutamine metabolism and usage in colorectal cancer cells, specifically HCT116 cells, would be compelling to further establish the respiratory profile. This could be achieved by depriving the cells of glutamine and performing carbon flux to determine whether there is a change in the input of carbons in the TCA cycle as well as a difference in glucose usage as measured by BioProfile Analyzer. If these cells do indeed depend on glutamine, the absence of this metabolite may synergize with deletion of TC-PTP to inhibit cellular respiration.

To try to better understand the mechanisms by which the cells decrease their mitochondrial usage for respiration, we performed western blotting against known substrates of TC-PTP as well as proteins involved in regulating cell survival or metabolism. We observed hyperphosphorylation of STAT1, STAT3 and p38 in CRC cells lacking TC-PTP. A report has previously established that constitutive activation of STAT3

can activate and maintain the Warburg effect and downregulate mitochondrial activity (188). In our system, we observe the same decrease in mitochondrial activity in TC-PTP-deficient cells since STAT3 is hyperphosphorylated, but we did not observe an increase in aerobic glycolysis. This may emphasize the importance of STAT3 expression levels and activity in cancer. Another study demonstrated that persistent STAT3 activation in CRC cells is associated with increased proliferation and tumour growth after stimulation with IL-6 (189). This is in concordance with the aforementioned study regarding the role of STAT3 in several types of cancer in promoting hallmarks of cancer, including promoting aerobic glycolysis and uncontrolled proliferation (50). Perhaps another event would be required in our TC-PTP-deficient STAT3-hyperactive colorectal cancer cell lines for glycolysis to be effectively favoured as a metabolic pathway and promote cancer cell growth.

We propose the following model: in absence of TC-PTP, STAT1, STAT3 and p38 are hyperphosphorylated. There is an upregulation in STAT1 and STAT3 dimers, which translocate to the nucleus and, in presence of another stress, further induce the transcription of genes involved in promoting mitochondrial respiration (*NDUFB5*) and reducing oxidative damage (*CAT* and *SOD1*) (**Figure 2.9**). To determine whether the phosphatase activity of TC-PTP is required for the regulation of cellular respiration, it would be important to inhibit the substrates of TC-PTP, such as STAT1 and STAT3, and establish whether the observed metabolic phenotype can be rescued. Considering that TC-PTP dephosphorylates STAT1 and STAT3, if their inhibition promotes mitochondrial respiration in these colorectal cancer cells, it may indicate that STAT1 and STAT3 are implicated in regulating cellular metabolism and that it is the phosphatase activity of

TC-PTP that regulates this process. Furthermore, p38 has been shown to promote STAT3 transcriptional activation upon stimulation with IL-6 (190). Additionally, through its regulation of the IL-6 signaling pathway, TC-PTP has also been shown to control gluconeogenesis, which is an important metabolic pathway used to generate glucose from non-carbohydrate carbon substrates such as lactate (191). As such, hyperphosphorylation of p38 upon inhibition of TC-PTP may further increase the activity of STAT3 in a positive feedback loop and induce metabolic gene transcription. In addition, although we have not demonstrated this yet, it is possible that TC-PTP would also dephosphorylate STAT1 and STAT3 that are localized to the mitochondria (**Figure 2.9**). To study this question, immunofluorescence could be performed to establish whether TC-PTP can localize to the mitochondria with STAT1 and STAT3 upon induction of stress. If this was the case, TC-PTP could directly modulate mitochondrial respiration by regulating STAT1 and STAT3, which have been shown to regulate complexes of the ETC.

No study as of yet has demonstrated that TC-PTP can localize to the mitochondria. However, some PTPs are known to be present in the mitochondria so it is not impossible considering its nuclear and cytoplasmic distribution that TC-PTP could also be present in this organelle. Furthermore, a high-throughput affinity-capture mass spectrometry study has identified a protein called TBF1M as an interacting partner of TC-PTP (192). TFB1M, for transcription factor B1, mitochondrial, is exclusively present in the mitochondria (193, 194). It is a nuclear-encoded mitochondrial biogenesis factor that is a dimethyltransferase for two adenosine residues of the mitochondrial 12S rRNA and thus regulates assembly

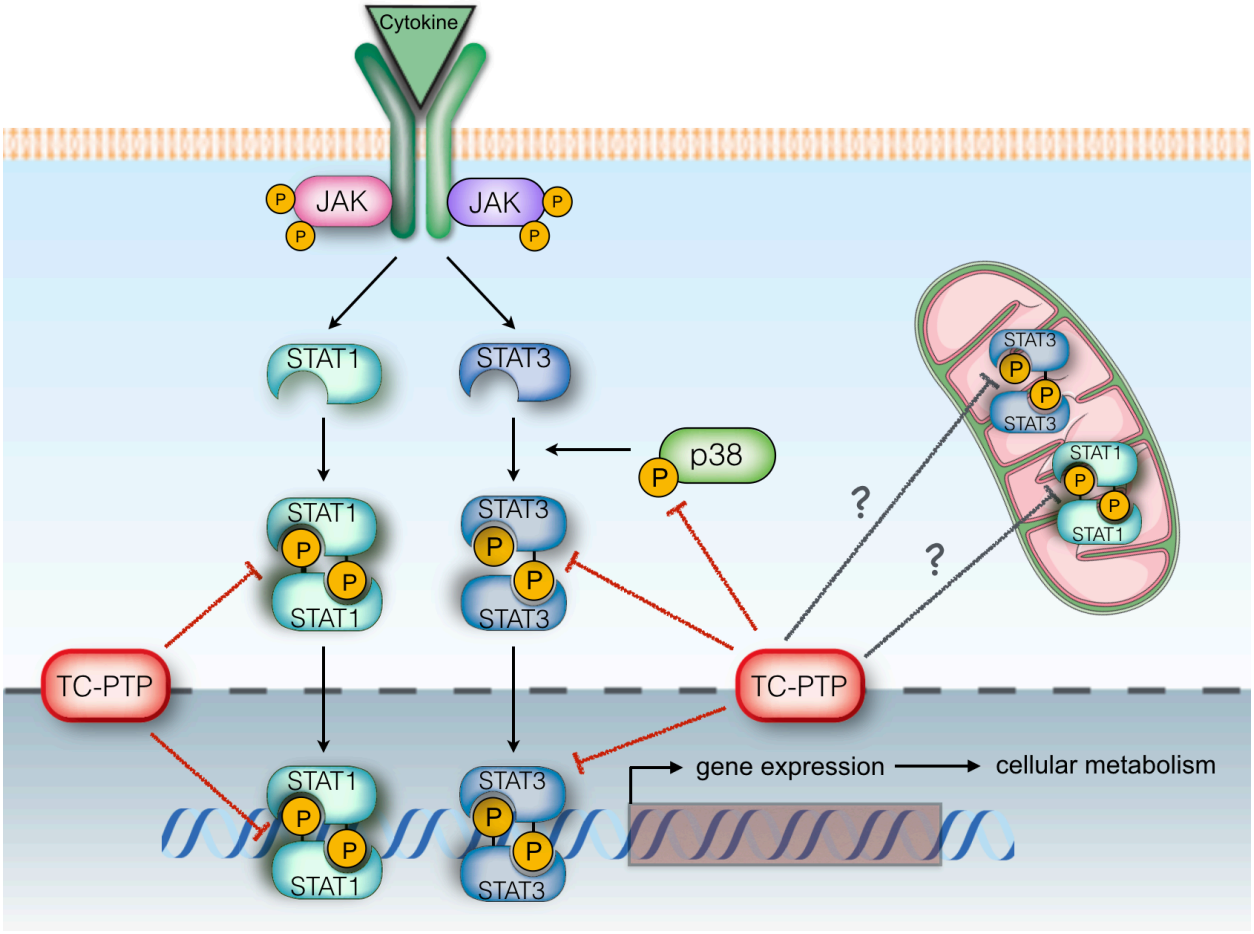

Figure 2.9: Proposed model. In colorectal cancer cells, TC-PTP deficiency leads to hyperphosphorylation and activation of STA1, STAT3 and p38. p38 can also regulate STAT3 activity. Upon phosphorylation, STAT1 and STAT3 dimers translocate to the nucleus and induce expression of genes that will result in decreased mitochondrial respiration. TC-PTP may potentially dephosphorylate STAT1 and STAT3 localized in the mitochondria and by doing so directly regulate mitochondrial respiration through modulation of the electron transport chain. Figure of mitochondria obtained from SMART (111).

or stability of the small subunit of the mitochondrial ribosome (195). TFB1M is also part of the basal mitochondrial transcription complex and is essential for the expression of mitochondrial genes. A report has shown that knockdown of TFB1M led to a reduction in mitochondrial protein synthesis in *Drosophila*, emphasizing its role in mitochondrial function and biogenesis (196). Several studies have demonstrated, both in mice and in

Drosophila, that inhibition of TFB1M resulted in decreased ATP production and oxygen consumption. In both models, absence of TFB1M resulted increased ROS production, and the cells were more sensitive to this oxidative damage due to mitochondrial dysfunction (193, 194). In mice, loss of TFB1M led to an increase in the number of mitochondria, but these had a disrupted architecture as observed by electron microscopy (194). These reports established that TFB1M is required to regulate mitochondrial function and provide resistance to oxidative stress. Could there be a TC-PTP-TFB1M-STAT3 complex in the mitochondria? It will be very important to validate the interaction between TC-PTP and TFB1M observed by high-throughput screening and to further investigate whether TC-PTP regulates TFB1M activity in the mitochondria. Considering the major effect that TC-PTP inhibition has on mitochondrial respiration, it would be intriguing to determine if the regulation of cellular metabolism is directly through TFB1M, STAT1 or STAT3. Observing the structure of mitochondria derived from TC-PTP knockout cells or mice by transmission electron microscopy would be very exciting as well.

It has been demonstrated that in colorectal cancer patients, the intensity and site of inflammation can lead to different outcomes. Local and more acute inflammatory responses have been associated with improved survival, whereas increased systemic inflammation has been linked with poor survival (197). The proposed mechanism in the study by Spitzner et al. implicated the activation of the IL-6/JAK/STAT3 pathway. They have shown that in CRC patients undergoing resection, the expression of STAT3 was associated with adverse host inflammatory responses along with a reduced survival, suggesting that the upregulation of STAT3 in tumours may be an important mechanism by which the tumour dysregulates the balance between local and systemic inflammatory

responses (197). In our study, we have established that TC-PTP negatively regulates the phosphorylation of STAT3. As such, controlled inhibition of TC-PTP by the use of pharmaceutical drugs could regulate STAT3 activity and may increase patient outcome and survival in the context of colorectal cancer or colitis-associated colorectal cancer. Based on the systemic mouse model and our data, a full deletion of TC-PTP would not be ideal as it would promote systemic inflammation and persistent STAT3 activation, but if we could dampen the activity of STAT3 to levels approaching more the phenotype observed in heterozygous mice, it might be beneficial for the patients. To study the biological relevance of TC-PTP loss in CRC growth, xenografts mouse models could be designed by injecting HCT116 colorectal cancer cells either wild-type or deficient for TC-PTP intrarectally and observing the initiation and progression of tumours in normal conditions or upon induction of inflammation with dextran sodium sulfate (DSS). This would allow us to observe whether tumourigenesis and colorectal cancer progression *in vivo* is actually controlled by the expression of TC-PTP.

In summary, we have shown with our human phosphatome screen that several PTPs are implicated in regulating the cellular metabolism of colorectal cancer cells by either dampening or promoting mitochondrial respiration. Out of the 24 PTP candidates identified, we confirmed with additional studies that TC-PTP is a positive regulator of mitochondrial respiration and may be a novel negative regulator of the Warburg effect. This correlates with most studies in the literature that classify TC-PTP as a tumour suppressor. This metabolic screen provided a wealth of data that could be used to study other PTP involved in modulating the Warburg effect in cancer.

CHAPTER 3

TC-PTP REGULATES THE POLARIZATION AND IMMUNE RESPONSE OF MACROPHAGES

Vinette, V., Zolotarov, Y., Insull, H. and Tremblay, M.L.

3.1 Introduction

An emerging hallmark of cancer is dysregulated cancer bioenergetics, which we studied in the previous chapter and established that TC-PTP positively regulates the metabolism of colorectal cancer cells, specifically by augmenting their use of mitochondria to respire. Another well-established characteristic of cancer cells is tumour-promoting inflammation (50). We are now beginning to understand that inflammation and metabolism are intimately linked. Indeed, several studies have reported that inflammatory and metabolic responses can induce each other. One group has demonstrated that joint inflammation can result from metabolic stress (198), and another showed that inflammation-induced obesity can stem from metabolic alterations (199). Metabolic stress can activate various pathways, such as NF-κB and HIF-1α, which in turn can upregulate distinct inflammatory pathways and ultimately lead to inflammation (200-204). Similarly, inflammation can itself induce metabolic changes. A recent study by Qu et al. demonstrated that cellular metabolism can be reprogrammed by chronic inflammation during inflammatory bowel diseases (IBDs) and colitis-associated colorectal cancer (CAC). Inflammation of the colon resulted in upregulation of key glycolytic enzymes through a STAT3/c-Myc signaling pathway (100).

We have previously demonstrated that systemic deletion of TC-PTP in mice is lethal at three weeks of age due to severe systemic inflammation caused in part by the upregulation of the cytokines iNOS, TNF-α and IFN-ɣ (77). We have also shown that mice that are heterozygous for TC-PTP are more susceptible to colitis induced by DSS than their wild-type counterparts (205), thus proposing a role for TC-PTP in inhibiting colitis. Furthermore, we have established that it regulates macrophage development and

activation through control of the CSF-1R receptor (85, 206). However, not much is known about the potential role of TC-PTP in regulating the inflammatory status of macrophages. Given the overt and dysregulated pro-inflammatory cytokine signaling present in TC-PTP knockout mice, we sought to determine whether this phosphatase could act as a switch to reprogram macrophage polarization. Considering that macrophages are the predominant immune cell type from the innate immune system present in various cancers, including colorectal cancer, understanding their polarization status and how this process can be regulated could shed light on some breakthroughs for macrophage-based cancer immunotherapy (135).

In our study, we demonstrated that the protein tyrosine phosphatase TC-PTP regulates the polarization of macrophages and their immune response. TC-PTP-deficient macrophages were more apt to be polarized towards pro-inflammatory M1 macrophages. In addition, loss of TC-PTP in these macrophages led to dysregulation of their transcriptome and aberrant induction of several pathways involved in promoting macrophage function, the immune response, and mitochondrial respiration. We have also established that myeloid-specific deletion of TC-PTP leads to a similar phenotype in macrophages than a systemic deletion, albeit less pronounced. Hence, deletion of TC-PTP solely in macrophages appears to be enough to induce inflammation, but this effect is increased when the microenvironment, such as other immune cells, is also deficient for TC-PTP. The implication is that TC-PTP promotes macrophage immune responses in both a cell-intrinsic and cell-extrinsic manner.

3.2 Materials and Methods

3.2.1 Mice

The generation of mice with a constitutive germline deletion of PTPN2 (PTPN2$^{-/-}$ mice, also known as TC-PTP$^{-/-}$) has been described previously (92). Heterozygous breeding pairs were used to ensure that wild-type, heterozygous and knockout littermates were obtained. We also generated a myeloid-specific TC-PTP mouse model using LysM-Cre mice (named PTPN2/LysM-Cre). For the latter model, mice heterozygous for Cre were crossed with whole-body TC-PTP heterozygous mice. For experimental purposes, we crossed heterozygous TC-PTP mice with one breeder being Cre$^{+/-}$. All mice were housed in a specific pathogen-free facility in sterile microisolator caging. Animal protocols were in accordance with the regulations of the Canadian Council on Animal Care and were approved by the McGill University animal care committee.

3.2.2. Isolation and stimulation of murine immune cells

Immune cells were harvested from two mouse models: whole-body (systemic) and LysM-Cre (myeloid-specific) deletion of TC-PTP. Monocytes, B cells and T cells were harvested from the bone marrow, spleen and thymus, respectively. Monocytes were either maintained as such or differentiated into macrophages by stimulating them with 30 ng/mL recombinant murine M-CSF (PeproTech) for 8 days, or into dendritic cells by stimulating them with 40 ng/mL recombinant murine GM-CSF (PeproTech) and 40 ng/mL recombinant murine IL-4 (PeproTech) for 6 days. For both macrophages and dendritic cells, cytokines were replenished every 3 days. Monocytes, macrophages and dendritic cells were grown in 10 cm^2 petri dishes in DMEM supplemented with 10% heat-inactivated fetal calf serum, 1% GlutaMAX, 1% sodium pyruvate, 1% Pen/Strep, 1% non-essential

amino acids and 0.1% β-mercaptoethanol in a 5% CO_2 incubator at 37°C. Macrophages were either maintained as naive macrophages, or polarized towards the M1 pro-inflammatory phenotype using 20 ng/mL recombinant murine IFN-ɣ (PeproTech) and 100 ng/mL lipopolysaccharides (LPS, *E. coli* O55:B5, Sigma-Aldrich), or towards the M2 anti-inflammatory phenotype using 20 ng/mL IL-4 for 24 hours. For downstream experiments such as qRT-PCR, western blotting and flow cytometry, monocytes and macrophages plated in a 10 cm^2 petri dish were harvested by washing twice with PBS and gently scraping with a cell scraper. Dendritic cells were collected by centrifuging the supernatant at 1,500 rpm at 4°C for 5 minutes and removing the supernatant. B cells isolated from the spleen were purified with an EasySep Mouse B cell negative selection isolation kit (STEMCELL Technologies) and T cells were isolated from the thymus and washed twice with PBS.

3.2.3 RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was isolated from macrophages using the TRIzol reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. RNA was quantified with NanoDrop and the quality of RNA was determined by running an RNA gel using 500 ng to 1 µg RNA on a 1% agarose gel. 1 µg of RNA was reverse-transcribed to cDNA using the SuperScript III Reverse Transcriptase Kit (Thermo Fisher Scientific), according to the manufacturer's protocol. qRT-PCR was performed with a LightCycler 480 (Roche) using SYBR Green Master Mix (Roche) according to the manufacturer's instructions. A panel of different housekeeping genes was tested with samples from different genotypes and polarization status, and *Actb* was determined to be the most accurate and reliable housekeeping gene. As such, it was used as a reference gene to normalize the relative

abundance of each gene of interest for all qRT-PCR experiments.

3.2.4 Western blotting

Total cell lysates (TCL) were prepared from macrophages, monocytes, dendritic cells, B cells or T cells using mRIPA buffer supplemented with EDTA-free protease inhibitor (Roche) and 2 mM sodium orthovanadate (Sigma-Aldrich). Protein levels were quantified by bicinchoninic acid (BCA) assay. 15 or 20 µg of each lysate was ran on 10% SDS-PAGE gels using the anti-mouse primary antibody TC-PTP (1/10, homemade clone 3E2), as well as the anti-rabbit primary antibodies STAT3 (1:1,000, Cell Signaling Technology), p-STAT3 (1:1,000, Tyr705, Cell Signaling Technology), p38 (1:1,000, Cell Signaling Technology), p-p38 (1:1,000, Thr180/Tyr182, Cell Signaling Technology) and vinculin (1:2,000, Sigma-Aldrich). The anti-rabbit primary antibody calnexin (1:50,000) was provided from J.J.M. Bergeron (McGill University), and secondary antibodies anti-rabbit and anti-mouse HRP (1:10,000) were obtained from Jackson ImmunoResearch. Each sample represents an individual mouse. Blots were imaged on the Mini-Medical Series imager (AFP Imaging).

3.2.5 Flow cytometry

Fc receptors on purified bone marrow-derived cells were blocked with anti-CD16 antibodies (BD Biosciences). Cell surface antigens were detected with the following antibodies diluted in PBS (Wisent) supplemented with 2.5% fetal calf serum: phycoerythrin (PE)-conjugated anti-F4/80, fluorescein isothiocyanate (FITC)-conjugated anti-CD11b, PE-Cy7-conjugated anti-CD206 and peridinin-chlorophyll protein (PerCP)-Cy5.5-conjugated anti-CD301 from BioLegend. For experiments requiring detection of intracellular antigens by flow cytometry, cells were then fixed and permeabilized, and

finally stained for the intracellular markers. NOS2 was detected as such using an allophycocyanin (APC)-conjugated antibody (Thermo Fisher Scientific). F4/80 and CD11b were used as general macrophages markers, NOS2 as an M1 macrophage-specific marker, and CD206 and CD301 as M2 macrophage-specific markers. Dendritic cells were stained with FITC-conjugated anti-CD11c and APC-conjugated anti-MHCII. Purified B cells were stained with APC-conjugated anti-B220 and FITC-conjugated anti-CD19, while T cells were stained with APC-Cy7-conjugated anti-CD4 and FITC-conjugated anti-CD8. All cells were stained with the Live/Dead fixable Aqua viability dye (Thermo Fisher Scientific). Samples were collected with a FACSCanto II instrument (BD Biosciences) and analyzed with FlowJo software (Tree Star).

3.2.6 Enzyme-linked immunosorbent assays (ELISAs)

Macrophages were harvested from the bone marrow of PTPN2/LysM-Cre wild-type (WT) and knockout (KO) mice. Cells were divided into three populations and plated in 10 cm^2 petri dishes: an unstimulated population of naive BMDMs (Mo), macrophages polarized towards the pro-inflammatory phenotype using IFN-ɣ and LPS for 24 hours (M1), and macrophages polarized towards the anti-inflammatory phenotype using IL-4 for 24 hours (M2). After cells were polarized for 24 hours, 2 mL of supernatant from each dish was collected, transferred to a microtube and centrifuged at 10,000 rpm at 4°C to remove cell debris. Clean supernatant was transferred to a fresh microtube and ELISA was performed according to the manufacturer's protocol to quantify the expression of the cytokines IFN-ɣ, IL-6 and TNF-α. Data were normalized to cell number.

3.2.7 RNA-sequencing and data analysis

RNA from bone marrow-derived macrophages was isolated with TRIzol and purified with PureLink DNase Set (Thermo Fisher Scientific). Samples were subjected to RNA-sequencing (RNA-Seq) on an Illumina HiSeq 2500. Transcript abundances were quantified with Kallisto v0.41.1 (207) using paired-end reads pseudo-aligned to the mouse transcriptome (GENCODE M17). Differential gene expression analysis was performed by importing Kallisto results with tximport v1.6.0 (208) followed by DESeq2 v1.18.1 (209). Genes were filtered based on adjusted p-value below 0.05 and log-transformed expression fold change of 2 or more. Ingenuity Pathways Analysis (IPA) and InnateDB were used for downstream analysis to identify pathways and biological processes that were enriched in specific populations.

3.2.8 Statistical analysis

All experiments conducted were analyzed using one-way analysis of variance (ANOVA) with a Dunnett post-test, unless otherwise noted. Other experiments, such as figures 3.4 and 3.10, were analyzed using a two-way analysis of variance (2way ANOVA) with a Tukey post-test.

3.3 Results

3.3.1 Generation of polarization gene panel for bone marrow-derived macrophages

To be able to study the role of TC-PTP in regulating the polarization of macrophages, it was first essential to optimize a protocol for their efficient polarization from naive (Mo) macrophages towards pro-inflammatory (M1) or anti-inflammatory (M2) macrophages (**Figure 1.6**). We also required a set of genes that would confidently allow us to determine

if the polarization process was successful. To do so, we thoroughly curated the literature to establish a group of 11 genes that were most often used to specifically characterize M1 or M2 macrophages derived from the bone marrow (210-217). It was important that we find genes that were efficient at differentiating M1 from M2 bone marrow-derived macrophages (BMDMs) as opposed to other types of macrophages such as those obtained from the spleen or the peritoneal cavity because the expression of M1- or M2-specific genes may be distinct in different immune compartments (211, 213).

We initially performed qRT-PCR with TC-PTP wild-type Mo, M1 and M2 macrophages to determine the expression of these polarization genes. Based on the literature, we selected *Ccr7*, *Cxcl11*, *Il12b*, *Nos2*, *Ptgs2* and *Tnf* as M1-specific genes, and *Arg1*, *Ccl24*, *Chil3*, *Retnla* and *Ptgs1* for M2-specific genes. We observed that all M1 genes analyzed were very good identifiers of M1 BMDMs as they were solely expressed in the M1 population and absent in the naive or M2 BMDMs (**Figure 3.1A**). We observed a slight expression of *Tnf* in the naive Mo BMDMs, but it was very low and absent in M2 cells, so we kept this gene in our panel. This confirmed that for our system, these genes were good and selective M1 markers. On the other hand, of the M2-specific genes tested, only half were efficient in differentiating M2 BMDMs from Mo or M1 cells. Although *Ccl24* and *Chil3* have been reported to be M2-specific genes in numerous studies (211, 214, 215), in our hands with our mouse model this was not the case (**Figure 3.1B**). Expression of these genes was very high in the M1 BMDMs, which was far from ideal since it will be crucial for downstream experiments to be able to separate these two populations. Hence, we decided to keep only the genes that were highly enriched in M2 BMDMs: *Arg1*, *Retnla* and *Ptgs1*. We thus established a macrophage polarization gene panel that we were very

confident in and would use for every experiment to confirm that the phenotype of the macrophages is what we expected. Of note, *Nos2* and *Retnla* were the most efficient M1- and M2-specific genes from our polarization panel, respectively.

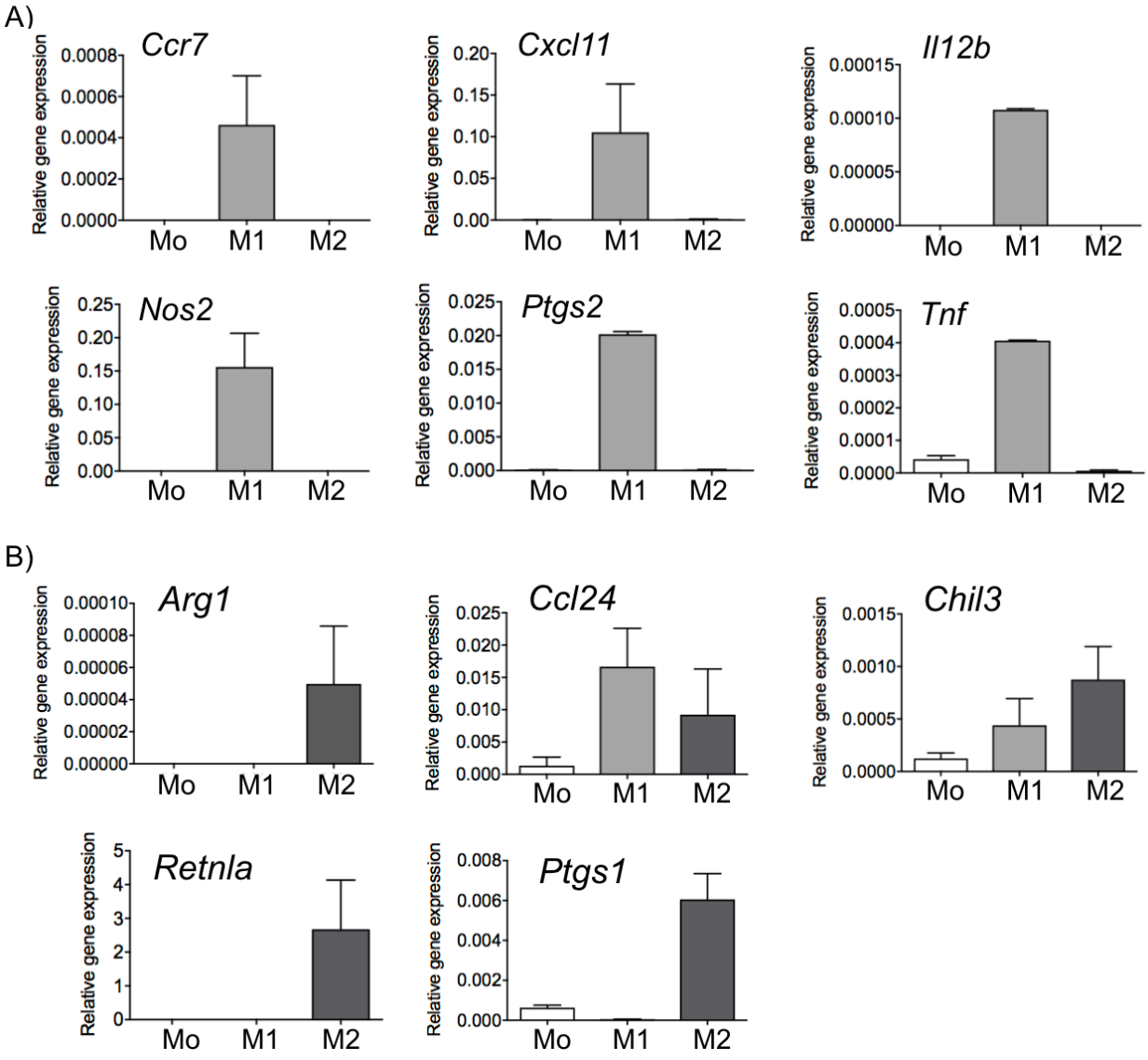

Figure 3.1: Validation of macrophage polarization gene panel. RNA was isolated from Mo, M1 and M2 BMDMs derived from wild-type mice. qRT-PCR was performed to validate our polarization gene panel for (A) M1-specific and (B) M2-specific genes. Data represent mean ± SEM of two experiments performed in triplicate. All samples were normalized to the reference gene *Actb*.

3.3.2 Validation of efficient bone marrow-derived macrophage polarization

After having established a panel of polarization genes that could be used to confidently differentiate between M1 and M2 macrophages, we proceeded to perform flow cytometry with Mo, M1 and M2 BMDMs derived from TC-PTP wild-type (WT), heterozygous (HET) and knockout (KO) mice. We first selected macrophages using CD11b and the pan-macrophage marker F4/80 (213, 218), then used NOS2 as our M1 marker (219, 220), and CD206 and CD301 as our M2 markers (213, 221, 222). We initially analyzed the different mouse genotypes separately to ensure that polarization was efficient independently of TC-PTP expression. The flow cytometry plots were quantified by the mean fluorescence intensity (MFI) of each marker. We observed a 2.5- to 4.5-fold increase of NOS2 in the M1 cells compared to both Mo and M2 populations. On the other hand, M2-polarized BMDMs displayed an upregulation of about 2- to 3-fold of the cell surface markers CD206 and CD301 compared to Mo and M1 macrophages. Although CD206 and CD301 are reported to be markers characteristic of M2 macrophages, they were also expressed at low levels in Mo and M1 macrophages, which may allude to the dynamic nature of macrophages. What is important is that these markers allow us to properly differentiate the M1 and M2 populations. These data show that TC-PTP BMDMs are able to undergo polarization towards the pro- (M1) or anti- (M2) inflammatory phenotype. Interestingly, NOS2 upregulation seemed to be further increased in TC-PTP-deficient M1 macrophages (**Figure 3.2C**) compared to WT (**Figure 3.2A**) and HET (**Figure 3.2B**) M1 BMDMs, as the relative expression of NOS2 in M1 macrophages was much higher compared to Mo or M2 macrophages in absence of TC-PTP.

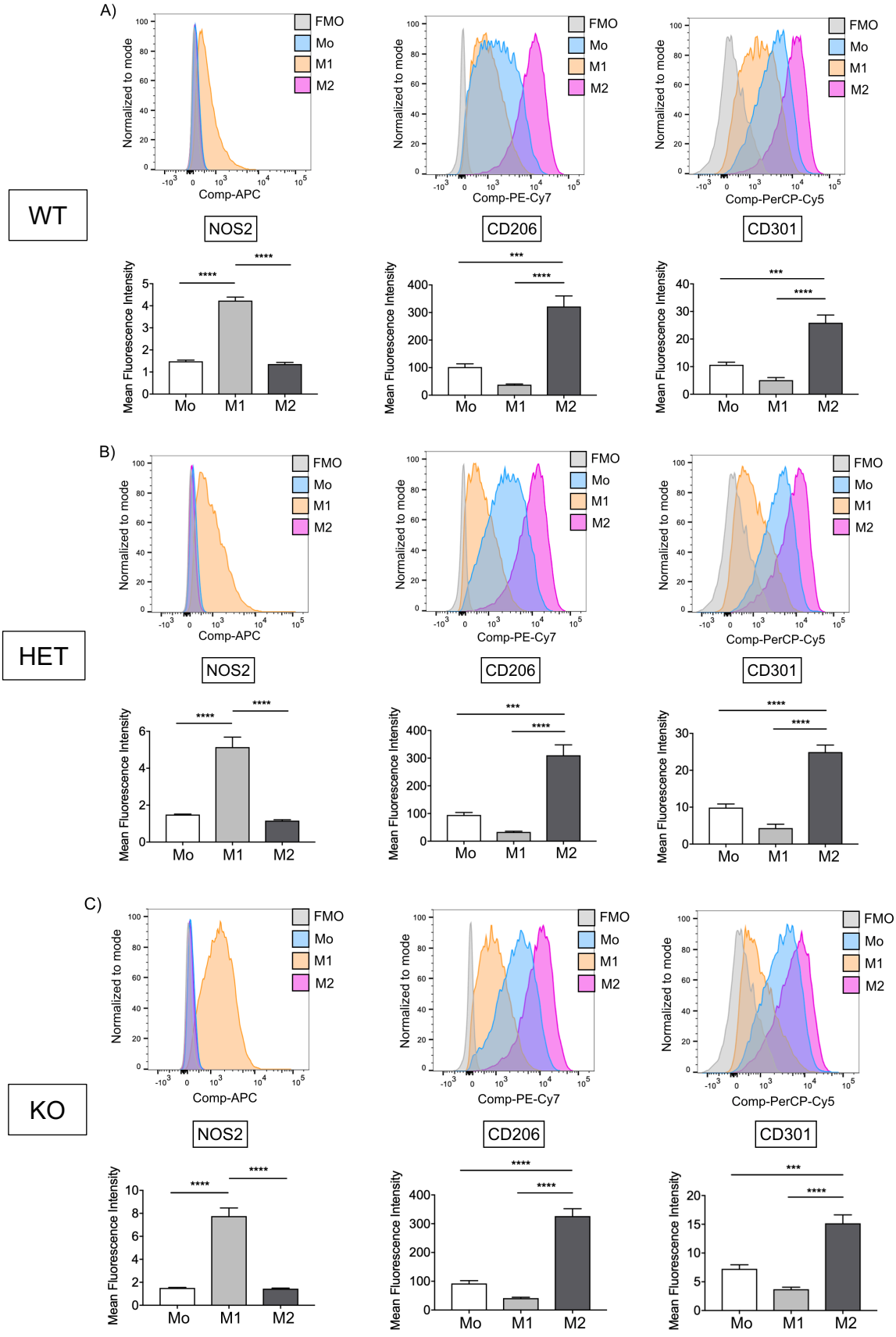
A)
B)
C)
WT
HET
KO
NOS2
CD206
CD301
FMO
Mo
M1
M2
Normalized to mode
Comp-APC
Comp-PE-Cy7
Comp-PerCP-Cy5
Mean Fluorescence Intensity
****
***

Figure 3.2: Efficient *ex vivo* polarization of bone marrow-derived macrophages. BMDMs from TC-PTP wild-type (WT), heterozygous (HET) and knockout (KO) mice were harvested and used as naive macrophages (Mo) or polarized towards pro-inflammatory (M1) or anti-inflammatory (M2) macrophages. Flow cytometry was performed with these BMDMs with the M1 marker NOS2 and the M2 markers CD206 and CD301. Associated with each plot is a bar graph of the mean fluorescence intensity (MFI) of each marker relative to the fluorescence minus one (FMO) control. We confirmed that this polarization strategy was effective for TC-PTP BMDMs from (A) WT, (B) HET and (C) KO mice. Data is the mean ± SEM of four mice per genotype and is representative of five independent experiments. *** $p < 0.005$; **** $p < 0.001$.

3.3.3 Systemic deletion of TC-PTP leads to upregulation of the pro-inflammatory marker NOS2 in macrophages

Our previous observation that TC-PTP M1 BMDMs had a higher upregulation of the M1 marker NOS2 led us to believe that TC-PTP could indeed have a role to play in regulating the polarization of macrophages. We performed additional flow cytometry experiments as aforementioned and further analyzed the expression of NOS2. First selecting viable cells that were singlets, we focused on the macrophage population by selecting F4/80$^+$ cells (**Figure 3.3A**). Of note, *ex vivo* differentiation and polarization of BMDMs from these mice always produce a very pure macrophage population, typically 98-99% F4/80$^+$ cells (**Figure 3.3A**). Focusing on the M1 population generated from TC-PTP WT, HET and KO mice, we analyzed the expression of NOS2 in these cells. Again, we observed an evident increase in the expression of NOS2 in TC-PTP-deficient macrophages compared to both wild-type and heterozygous mice (**Figure 3.3B**). This was repeated several times and summarized as the mean NOS2 MFI of 5 independent experiments (**Figure 3.3C**). These data led us to conclude that TC-PTP does indeed regulate the polarization of macrophages. The inhibition of this phosphatase favours the polarization of macrophages towards the M1 pro-inflammatory phenotype.

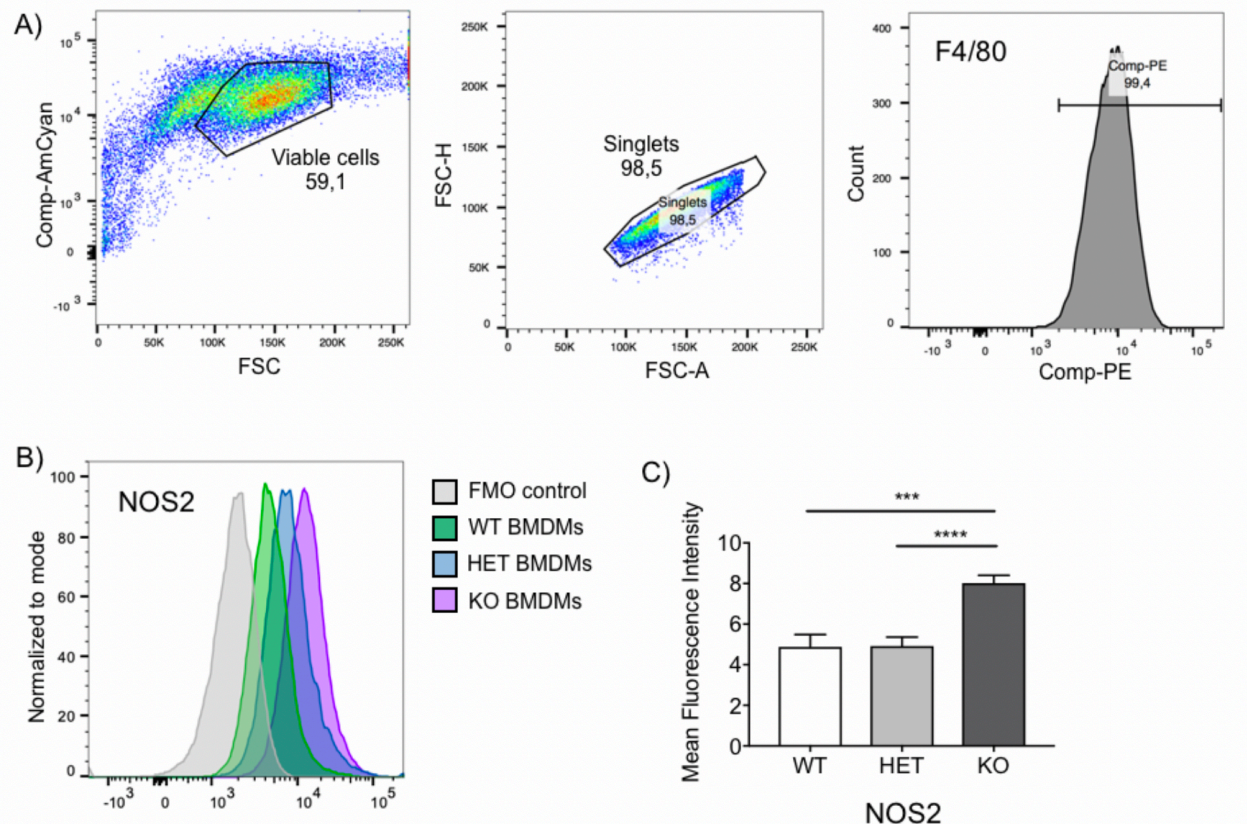

Figure 3.3: Increased expression of M1 marker NOS2 in TC-PTP-deficient macrophages. BMDMs were harvested from TC-PTP wild-type (WT), heterozygous (HET) and knockout (KO) mice and were polarized towards M1 macrophages. Flow cytometry was performed using the M1 marker NOS2. (A) Gating strategy to select live cells that are singlets and are positive for the pan macrophage marker F4/80. (B) Flow cytometry plot of M1 BMDMs from WT, HET and KO mice stained with NOS2. Data is the mean ± SEM of 3 to 6 mice per genotype and is representative of five independent experiments. (C) Summary MFI of NOS2 M1 marker. Data represent mean ± SEM of 3 to 6 mice per genotype and is the summary NOS2 expression of five independent experiments combined. *** $p < 0.005$; **** $p < 0.001$.

3.3.4 Inhibition of TC-PTP alters the gene expression of certain polarization genes that are characteristic of M1 or M2 macrophages

To further investigate the role of TC-PTP in promoting M1 polarization and to try to understand the mechanism by which this occurs, we performed qRT-PCR with Mo, M1 and M2 macrophages to analyze the expression of polarization genes from our pre-established panel. While we observed no difference in the gene expression of *Nos2*, we

did see a modulation of other M1-specific genes. *Cxcl11* was upregulated in M1 KO BMDMs compared to WT and HET cells, while *Il12b* expression was downregulated (**Figure 3.4A**). It seemed curious that the expression of various M1 genes would be modulated differently while others were completely unaffected by the absence of TC-PTP. Nonetheless, this may be due to distinct physiological functions. CXCL11 is a chemokine whose expression is strongly induced by IFN-ɣ and IFN-α (223, 224). Given that systemic TC-PTP knockout mice have a hyperproduction of IFN-ɣ that can lead to the activation of different immune cells (77, 92, 206), this could explain the observed transcriptional upregulation of *Cxcl11*. On the other hand, not as much is known regarding the role of IL-12 p40 (encoded by *Il12b*) in the immune response, apart from the fact that it is expressed by activated macrophages. Just like M1 genes, we found that the expression of distinct M2 genes was not regulated in the same manner. Only *Arg1* was modulated, and its expression was increased about 10-fold in KO M2 BMDMs compared to WT cells and 3-fold compared to HET BMDMs (**Figure 3.4B**). Other M2 genes did not vary in expression in a TC-PTP-dependent manner. These distinct modulations of polarization genes based on the expression of TC-PTP may also be explained by the innate plasticity of macrophages. To determine whether loss of TC-PTP promotes commitment to either macrophage subset, and not only the pro-inflammatory subset, it would be necessary to look at the protein levels of M2-specific genes, such as arginase 1 and IL-10, in these cells.

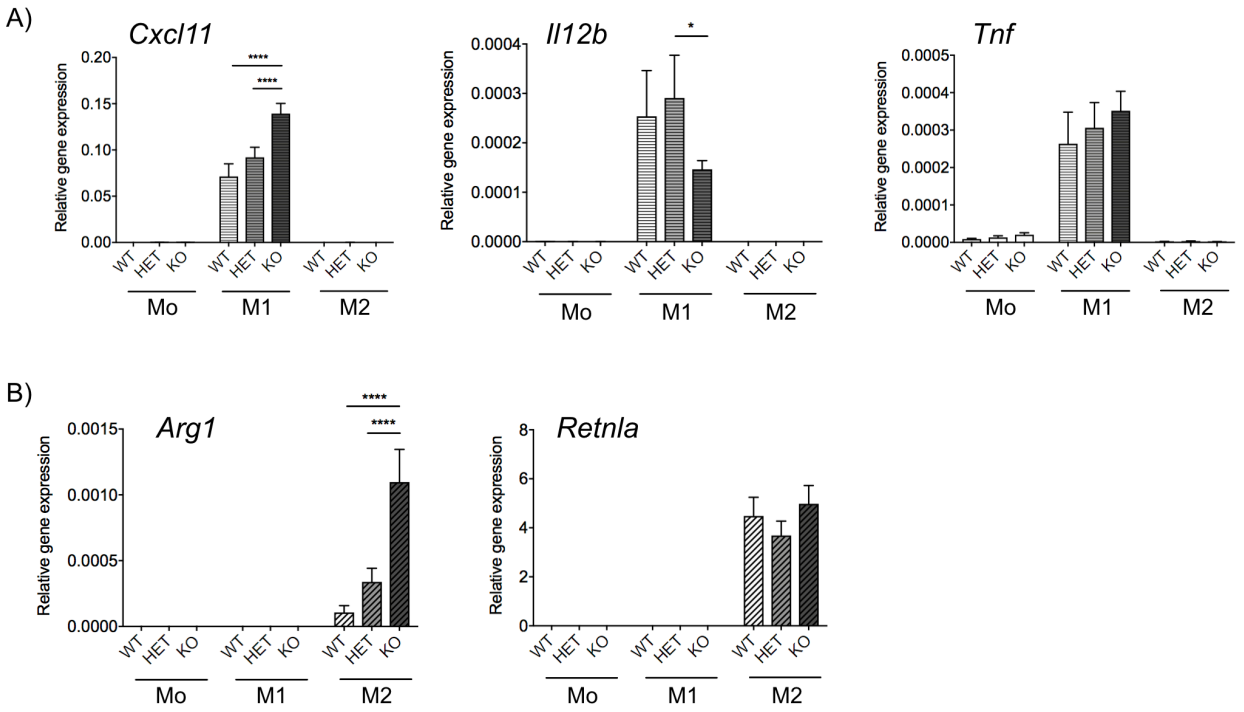

Figure 3.4: Loss of TC-PTP in M1 and M2 macrophages leads to differential expression of polarization genes. RNA was isolated from Mo, M1 and M2 BMDMs derived from TC-PTP wild-type (WT), heterozygous (HET) and knockout (KO) mice. qRT-PCR was performed using (A) M1 and (B) M2 polarization genes from our panel. Data represent mean ± SEM of 8 to 12 mice per genotype combined from three independent experiments and were normalized to the reference gene *Actb*. Data was analyzed using a 2way ANOVA with a Tukey post-test. * $p < 0.05$; **** $p < 0.001$.

3.3.5 Loss of TC-PTP in unstimulated macrophages results in changes in their transcriptome

So far, we have discovered that TC-PTP-deficient macrophages may be more prone to be polarized towards the M1 pro-inflammatory phenotype and favour a Th1 response *in vivo* based on the data observed *ex vivo* at the protein (**Figure 3.3**) and mRNA (**Figure 3.4**) levels. This discovery made us wonder whether the systemic deletion of TC-PTP would have a more profound effect on the transcriptome of macrophages that could explain their inflammatory status and the overt phenotype observed in mice (77, 92). To study this question, we performed RNA-Seq using BMDMs harvested from TC-PTP WT,

HET and KO mice that were polarized towards M1 or M2 macrophages. Subsequent analysis of our RNA-Seq samples provided us with a wealth of information concerning the potential effects of TC-PTP expression in macrophages of different immune phenotypes. Data presented will be divided by the type of macrophage inflammatory status: naive (Mo), pro-inflammatory (M1) and anti-inflammatory (M2). For each population, we identified a set of genes that were significantly downregulated in KO BMDMs compared to both WT and HET BMDMs.

When analyzing Mo macrophages, we determined that 103 genes were solely present in KO macrophages and 98 genes were only expressed in WT macrophages, while 188 transcripts were common to both populations (**Figure 3.5A**). We noted a similar pattern when comparing KO to HET macrophages (**Figure 3.5B**) and observed no significant differences between WT and HET BMDMs. We next performed gene ontology (GO) enrichment analysis to identify potential pathways and biological processes that could be over-represented depending on the expression of TC-PTP. By comparing the counts of the significantly upregulated or downregulated transcripts, we were able to identify several pathways that were regulated by TC-PTP. For instance, we noted an upregulation of genes involved in IFN-ɣ signaling, ATP binding as well as metabolic pathways in KO macrophages compared to WT cells (**Figure 3.5A**). Similar pathways were upregulated in TC-PTP-deficient macrophages compared to HET, except for the metabolic pathways (**Figure 3.5B**). These data propose that hemi-deficiency of TC-PTP is sufficient to alter the transcriptome of M1-polarized macrophages, and that modulation of several genes is affected by the precise expression level of TC-PTP, whether it be completely absent, present or partially present. In addition, the full deletion of TC-PTP

appears to lead to more changes, both quantitatively and qualitatively, and the genes affected may thus affect the regulation or expression of other genes.

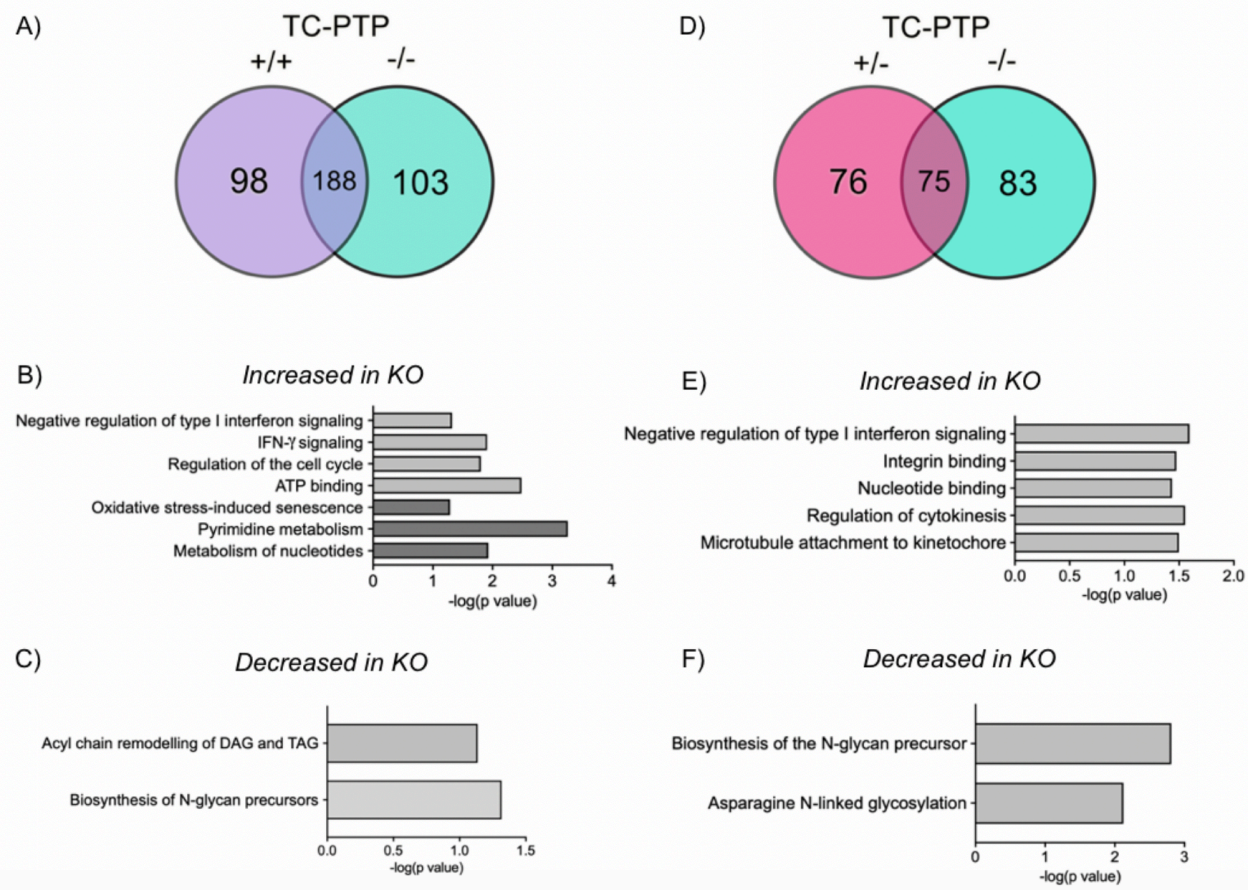

Figure 3.5: Altered transcriptome in Mo TC-PTP-deficient macrophages. RNA was purified from BMDMs harvested from TC-PTP wild-type (WT), heterozygous (HET) and knockout (KO) mice. RNA-Seq was performed using Illumina HiSeq 2500 and data was analyzed to compare the different populations. Venn diagrams represent genes that are exclusively upregulated in each population, while the intersection represents genes that are common to both. Gene ontology (GO) enrichment analysis was performed to identify pathways and biological processes enriched in certain populations, particularly (A) comparing WT to KO BMDMs, and (B) comparing HET to KO BMDMs. Data represent 3 mice per genotype.

We next sought to determine the top genes that were most dysregulated upon TC-PTP inhibition. To do so, we compiled a list of genes that were downregulated in KO BMDMs and calculated the adjusted log2(fold change) of the average transcripts of WT

and HET BMDMs populations compared to KO macrophages (**Table 3.1**). The top gene hits were the same in WT and HET macrophages compared to KO BMDMs. Interestingly, comparing the most upregulated genes in KO BMDMs versus WT or HET BMDMs indicated that there was no concordance between these two gene sets.

Table 3.1: List of 15 most downregulated genes in TC-PTP-deficient Mo macrophages

Gene name	Description	log_2(fold change)	
		WT	HET
Ftl1	ferritin light chain 1	29.84	29.37
Epb41	erythrocyte membrane protein band 4.1	24.08	23.29
Rpl22l1	ribosomal protein L22 like 1	23.87	23.09
Ptms	parathymosin	11.13	10.75
Ing5	inhibitor of growth protein 5	11.08	9.62
Cnpy3	canopy FGF signaling regulator 3	10.57	10.46
Ifngr2	interferon gamma receptor 2	10.24	10.28
Ncor1	nuclear receptor co-repressor 1	10.05	10.42
Slc35c2	solute carrier family 35, member C2	9.91	9.68
Brap	BRCA1 associated protein	9.65	9.29
Mrpl2	mitochondrial ribosomal protein L2	9.55	8.17
Zfp275	zinc finger protein 275	9.53	9.67
Rps10	ribosomal protein S10	8.57	8.41
Ankrd27	ankyrin repeat domain-containing protein 27	8.49	7.94
Rps15a	40S ribosomal protein S15a	8.03	7.94

3.3.6 Inhibition of TC-PTP in M1 macrophages induces aberrant transcription of genes involved in regulating the immune response and cellular metabolism

RNA-Seq analysis made evident that the transcriptome of M1 macrophages was strongly dysregulated in absence of TC-PTP. We established that 58 genes were only present in KO macrophages when comparing to the wild-type cells, while 130 genes were entirely absent in KO macrophages. 788 genes were significantly modulated in either population but were present in both (**Figure 3.6A**). We observed similar yet fewer

changes when comparing KO to HET BMDMs (**Figure 3.6B**). Upon GO enrichment analysis, we discovered that very similar pathways were altered in KO macrophages in relation to both WT or HET macrophages. In both cases, loss of TC-PTP resulted in over-representation of antigen processing and presentation, MHC class II protein complex and immune response pathways, while pathways involving cellular response to IL-1, TNF, IFN-α and viruses were downregulated in KO macrophages (**Figure 3.6**). What we found particularly intriguing was that inhibition of TC-PTP in M1 macrophages resulted in the upregulation of several metabolic pathways, such as oxidative phosphorylation, ATP synthesis-coupled electron transport and NADH dehydrogenase activity. It thus appears that TC-PTP deficiency in macrophages not only modulates the transcription of genes involved in the immune response and macrophage function, but also those involved in metabolism, seemingly favouring mitochondrial respiration.

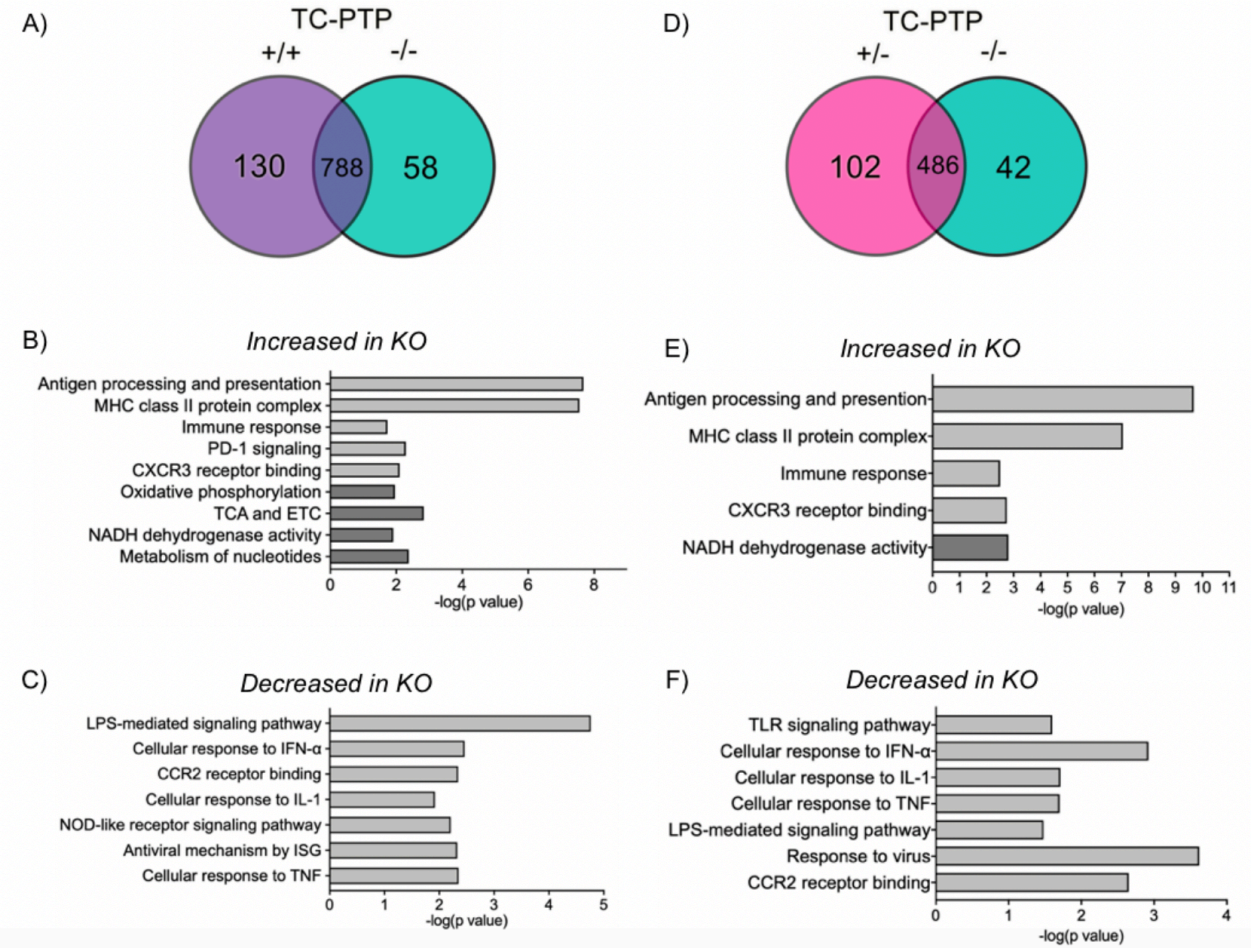

Figure 3.6: Inhibition of TC-PTP in M1 macrophages leads to aberrant transcriptome and upregulation of inflammatory and metabolic pathways. BMDMs were harvested from TC-PTP wild-type (WT), heterozygous (HET) and knockout (KO) mice and polarized towards M1 macrophages using IFN-ɣ and LPS for 24 hours. RNA was purified from these BMDMs and RNA-Seq performed using Illumina HiSeq 2500. Venn diagrams represent genes exclusively upregulated in each population while the intersection represents genes that are common to both. Gene ontology (GO) enrichment analysis was performed to identify pathways and biological processes enriched in certain populations, particularly (A) comparing WT to KO M1 BMDMs, and (B) comparing HET to KO M1 BMDMs. Data represent 3 mice per genotype.

The list of top 15 genes downregulated in knockout macrophages compared to WT and HET macrophages provided additional knowledge on the role of TC-PTP in regulating the transcriptome of M1 macrophages (**Table 3.2**). We recognized that the most downregulated gene in knockout M1 BMDMs, *Ftl1*, was the same as in Mo macrophages.

This was intriguing because its log-transformed fold change was about 27-fold higher in WT and HET macrophages in both Mo and M1 populations compared to KO macrophages. *Ftl1* is known to be implicated in regulating iron homeostasis and may as such regulate macrophage function (225, 226). We did not find any concordance in the genes upregulated in KO macrophages if we compared them to WT or HET BMDMS.

Table 3.2: List of 15 most downregulated genes in TC-PTP-deficient M1 macrophages

Gene name	Description	log_2(fold change)	
		WT	HET
Ftl1	ferritin light chain 1	26.93	26.05
Ifit1bl1	interferon induced protein with tetratricpeptide repeats 1B like 1	24.52	23.79
Col4a1	collagen type IV, alpha 1	22.29	22.14
Acvrl1	activin A receptor, type II-like 1	9.86	10.02
Slc15a4	solute carrier family 15, member 4	9.48	8.82
Clip1	CAP-Gly domain-containing linker protein 1	8.88	8.81
Ccdc86	coiled-coil domain containing protein 86	8.76	8.44
Ttyh3	tweety family member 3	8.64	8.56
C2cd5	C2 calcium-dependent domain containing 5	8.43	8.56
Otud5	OTU domain containing protein 5	8.37	8.29
Stxbp1	syntaxin binding protein 1	8.35	9.37
Adck1	aarF domain containing kinase 1	8.24	7.99
Slco4a1	solute carrier organic anion transporter family, member 4a1	8.23	7.87
Tnfrsf26	tumor necrosis factor receptor superfamily, member 26	7.63	7.50
Nrros	negative regulator of reactive oxygen species	7.59	7.43

3.3.7 The transcriptome of M2 macrophages is affected by the loss of TC-PTP

Analysis of the dataset obtained from M2 macrophages revealed much fewer changes in their transcriptome compared to either Mo or M1 populations. We determined that 60 genes were exclusively present in KO BMDMs while 81 were completely absent in KO BMDMs compared to WT cells (**Figure 3.7A**). As expected, the partial deletion of TC-PTP in HET macrophages resulted in even fewer changes when compared to KO macrophages (**Figure 3.7B**). GO enrichment analysis indicated that when comparing WT

and KO BMDMs, no pathways were significantly enriched in TC-PTP-deficient cells, while few pathways were upregulated in WT BMDMs, such as cellular response to extracellular stimulus and CD28 co-stimulation. CD28 co-stimulation promotes macrophage suppression of T-cells (227, 228), so absence of TC-PTP may serve to keep this process in check and ensure that T cells are functional. In contrast, many pathways were significantly upregulated in KO BMDMs when comparing to HET cells, such as various cell cycle or DNA damage checkpoint pathways (**Figure 3.7B**).

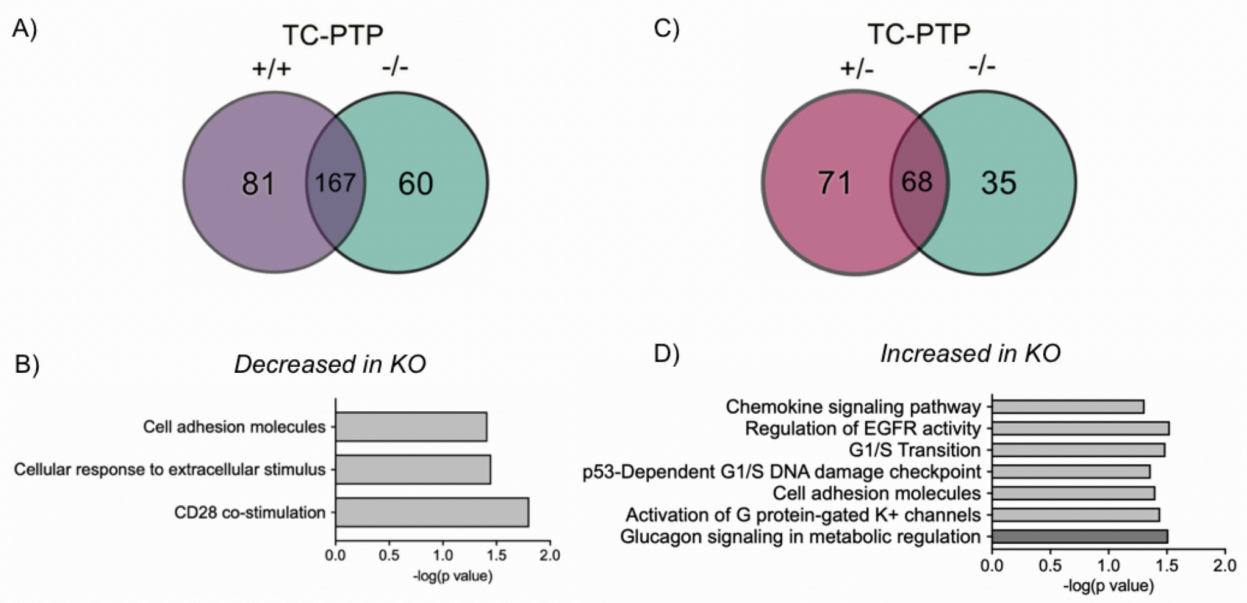

Figure 3.7: Deletion of TC-PTP in M2 macrophages modulates their transcriptome. BMDMs were harvested from TC-PTP wild-type (WT), heterozygous (HET) and knockout (KO) mice and polarized towards M2 macrophages using IL-4 for 24 hours. RNA was purified from these BMDMs and RNA-Seq was performed using Illumina HiSeq 2500. Venn diagrams represent genes exclusively upregulated in each population while the intersection represents genes that are common to both. Gene ontology (GO) enrichment analysis was performed to identify pathways and biological processes enriched in certain populations, particularly (A) comparing WT to KO M2 BMDMs, and (B) comparing HET to KO M2 BMDMs. Data represent 3 mice per genotype.

While compiling a list of the top 15 genes that were downregulated in KO macrophages, we noticed that three of these were zinc finger domains (**Table 3.3**). Whether this has any physiological relevance would need to be studied. Intriguingly, just like in the Mo and M1 macrophages, when we looked at the genes upregulated in KO BMDMs, we were unable to observe a consistent pattern when comparing to either WT or HET BMDMs. The fact that we observed this in macrophages with different inflammatory phenotypes made us wonder why this was the case. Additional studies will be required to determine whether this is a coincidence or physiologically relevant.

Table 3.3: List of 15 most downregulated genes in TC-PTP-deficient M2 macrophages

Gene name	Description	log_2(fold change)	
		WT	HET
Mdfic	MyoD family inhibitor domain containing	23.05	21.80
Cln3	ceroid lipofuscinosis, neuronal 3, juvenile	22.64	21.77
Rps12-ps3	ribosomal protein S12, pseudogene 3	12.08	12.68
Acp5	acid phosphatase type 5, tartrate-resistant	9.47	9.19
Zscan21	zinc finger and SCAN domain containing 21	9.01	7.85
H2-Ab1	histocompatibility 2, class II antigen A, beta 1	8.67	7.87
Eml4	echinoderm microtubule associated protein like 4	8.67	8.77
Lzts2	leucine zipper, putative tumor suppressor 2	8.62	7.92
Zmym5	zinc finger, MYM-type 5	8.34	9.77
Trim34a	tripartite motif-containing 34A	8.32	8.63
Apoe	apolipoprotein E	8.18	7.75
Mfsd13a	major facilitator superfamily domain containing 13a	8.15	8.28
Pus1	pseudouridine synthase 1	8.03	8.53
Zbtb7b	zinc finger and BTB domain containing 7B	6.93	6.53
Relb	avian reticuloendotheliosis viral (v-rel) oncogene related B	5.67	5.83

3.3.8 Identification of novel macrophage polarization markers

We have previously validated by qRT-PCR several M1-specific (*Nos2*, *Cxcl11*, *Ccr7*, *Ptgs2*, *Il12b*, *Tnf*) and M2-specific (*Retnla*, *Arg1*, *Ptgs1*) polarization markers based on a panel of genes identified from reviewing the literature (210-217) (**Figure 3.1**). RNA-Seq

further confirmed that these M1 and M2 polarization genes were highly upregulated in the expected populations. Unexpectedly, RNA-Seq may have led us to discover new polarization genes. Upon a more in-depth analysis, we discovered several genes that were only present in M1 macrophages and others that were only present in M2 macrophages. The 5 most upregulated of these genes for M1 macrophages were *Mx1*, *Serpinb9*, *Il15ra*, *Gbp4* and *Wnk2* (**Table 3.4**). These genes were only expressed in TC-PTP wild-type, heterozygous and knockout M1 macrophages, and completely absent in Mo or M2 cells. Curiously, not much is known about these genes in the context of macrophages or even the immune response, apart from the fact that *Mx1* is induced by type I and II interferons such as IFN-ɣ (229, 230).

On the other hand, much more was known about the potential novel M2 polarization genes established through our RNA-Seq dataset. The 5 most highly upregulated M2-specific genes were *Scn3a*, *Msx3*, *Cldn11*, *Tmem26* and *Pdcd1lg2* (**Table 3.4**). Although they do not seem to be used as polarization markers as of yet, some have been shown to play a role in macrophage function or polarization. For instance, *Scn3a*, *Msx3*, *Cldn11* and *Pdcd1lg2* are all genes that have been shown to be upregulated in IL-10- or IL-4-stimulated bone marrow-derived macrophages (231-233). Interestingly, *Msx3* has also been demonstrated to suppress pro-inflammatory pathways through the activation of PPARɣ and STAT6 anti-inflammatory pathways, which in turn suppress pro-inflammatory pathways such as NF-κB (234-236). As an indication that these may be true polarization markers, *Msx3* has previously been shown to switch the polarization of microglia, macrophages in the brain, to M2 microglia (234). Hence, by performing RNA-Seq on TC-PTP wild-type, heterozygous and knockout macrophages polarized towards

M1 or M2 macrophages, we have established a list of potential novel polarization genes that could be used experimentally to validate efficient polarization of macrophages. Additional experiments would need to be performed with more samples to confirm their efficacy and specificity, but these genes could prove useful in the case where previously established M1- or M2-specific genes may be absent or dysregulated in a given model.

Table 3.4: Novel polarization markers for bone marrow-derived macrophages

Known polarization genes		Novel polarization genes	
M1	**M2**	**M1**	**M2**
Nos2	*Retnla*	*Mx1*	*Scn3a*
Cxcl11	*Arg1*	*Serpinb9*	*Msx3*
Ccr7	*Ptgs1*	*Il15ra*	*Cldn11*
Ptgs2		*Gbp4*	*Tmem26*
Il12b		*Wnk2*	*Pdcd1lg2*

RNA-Seq analysis of the different populations of macrophages with various levels of TC-PTP expression provided us with a tremendous amount of knowledge that will require more time to sift through and understand the consequences of such transcriptomic modifications. Nonetheless, we have acquired some insights into the role of TC-PTP in regulating different immune and metabolic pathways, and some of these will be investigated experimentally.

3.3.9 Generation and validation of PTPN2/LysM-Cre mouse model

Thus far, we have demonstrated that systemic germline deletion of TC-PTP in mice produces macrophages that have a more pro-inflammatory signature and are more apt at being polarized towards this M1 phenotype. While the transcriptome of naive and

anti-inflammatory BMDMs were affected by the inhibition of TC-PTP, the transcriptome of pro-inflammatory macrophages seems to be aberrantly dysregulated. TC-PTP-deficient M1 macrophages displayed an upregulation of pathways involved in regulating the immune response and activation, as well as several metabolic pathways (**Figure 3.6**). These data left us wondering whether a constitutive deletion of this phosphatase was required for the observed phenotype in macrophages, or whether TC-PTP could also have a cell intrinsic effect. To answer this question, we generated a mouse model where deletion of TC-PTP would be restricted to myeloid cells, notably macrophages, neutrophils and dendritic cells. This is the LysM-Cre mouse model often used to study the function of macrophages. Although other cells from the myeloid compartment may be affected, macrophages are typically the major myeloid cell type affected by the Cre recombinase inserted into the lysozyme 2 gene (237-240). We generated PTPN2/LysM-Cre mice and aimed to validate the efficient knockdown of TC-PTP exclusively in myeloid cells.

We first performed flow cytometry to ensure that cells harvested and purified from LysM-Cre mice were the expected populations. Using antibodies specific for monocytes (F4/80 and CD11b), macrophages (F4/80 and CD11b), dendritic cells (CD11c and MHCII), B cells (B220 and CD19) and T cells (CD8 and CD4), we confirmed that we had the proper immune cell populations since they expressed the traditional cell surface markers. Monocytes and macrophages were F4/80 and CD11b double-positive ($F4/80^{+}$ $CD11b^{+}$) (241, 242). For dendritic cells, there was a larger $CD11c^{+}$ $MHCII^{HIGH}$ population and a smaller $CD11c^{+}$ $MHCII^{LOW}$ population. The latter population represents immature or less activated dendritic cells (243, 244). B cells were predominantly B220

and CD19 double-positive ($B220^+$ $CD19^+$) while for T cells we observed three distinct populations: a small proportion of $CD8^+$ T cells, some $CD4^+$ T cells and the majority being CD8 and CD4 double-positive cells ($CD8^+$ $CD4^+$) (**Figure 3.8A**). These three distinct populations reflect the different states and types of T cells that can arise from the thymus, with the $CD8^+$ $CD4^+$ being immature T cells which can later mature into $CD4^+$ helper T cells or $CD8^+$ cytotoxic (killer) T cells (245-247). We next performed western blotting to determine the expression of TC-PTP in these distinct immune cells. Since the LysM-Cre mouse model only targets cells from the myeloid compartment, TC-PTP should only be deleted in monocytes, macrophages and dendritic cells. Indeed, inhibition of TC-PTP was observed only in these immune cells in a dose-dependent manner, from knockout (fl/fl $Cre^{+/-}$ and fl/fl $Cre^{-/-}$) to heterozygous (fl/wt $Cre^{+/-}$) to wild-type (wt/wt $Cre^{+/-}$ and wt/wt $Cre^{-/-}$) mice (**Figure 3.8B**). Additionally, deletion of TC-PTP seemed to be more efficient in monocytes and macrophages compared to dendritic cells, which has been previously reported in different LysM-Cre mouse models (237, 239, 240). We saw normal expression of TC-PTP in B cells and T cells, regardless of the genotype of the mice. These data confirm that TC-PTP deletion in our PTPN2/LysM-Cre mice is only observed in myeloid cells, but more so in macrophages and monocytes.

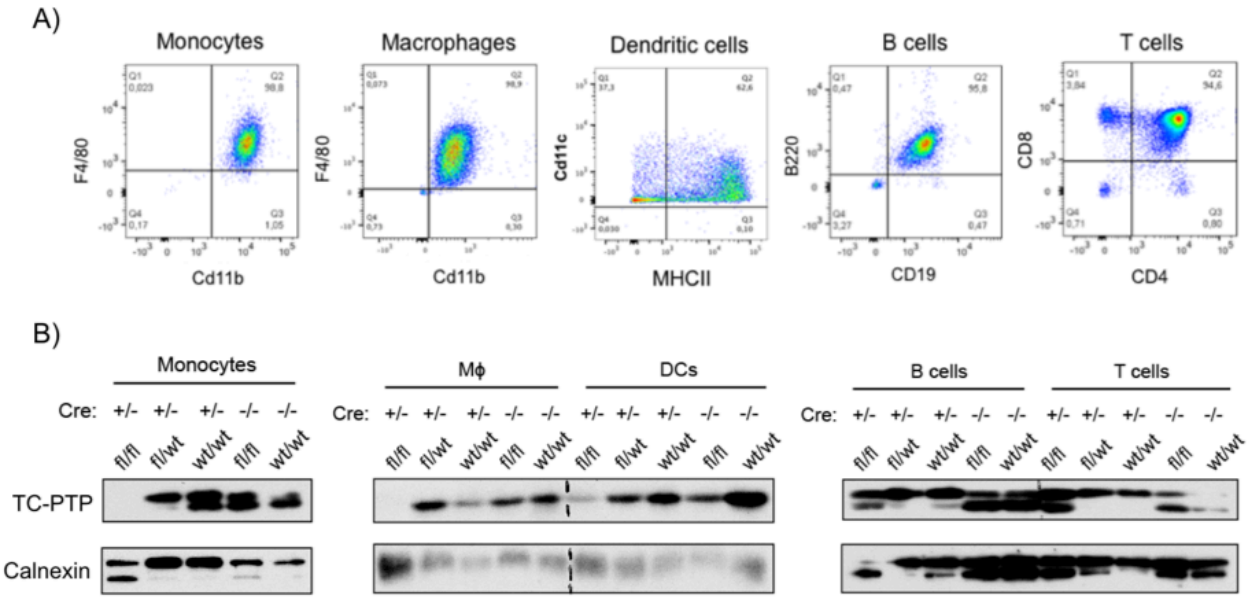

Figure 3.8: Validation of PTPN2/LysM-Cre mouse model by flow cytometry and western blot. Cells were isolated from the bone marrow, spleen and thymus of TC-PTP wild-type (wt/wt Cre$^{+/-}$ or wt/wt Cre$^{-/-}$), heterozygous (fl/wt Cre$^{+/-}$) or knockout (fl/fl Cre$^{+/-}$ or fl/fl Cre$^{-/-}$) mice to isolate monocytes, B cells and T cells, respectively. A subset of monocytes was differentiated either into macrophages with M-CSF or into dendritic cells with GM-CSF and IL-4. (A) Flow cytometry was performed to determine the purity of the immune cell populations. (B) Total cell lysate (TCL) was prepared from these cells, and western blotting was performed with 20 µg of TCL. Blots are representative of two independent experiments.

3.3.10 Inhibition of TC-PTP in macrophages derived from LysM-Cre mice causes hyperphosphorylation of STAT3

After validating that the PTPN2/LysM-Cre mice that we generated displayed a deletion of TC-PTP only in myeloid cells, we wanted to look at downstream signaling pathways that could be affected in these cells. Using macrophages derived from wild-type (WT, wt/wt Cre$^{+/-}$) and knockout (KO, fl/fl Cre$^{+/-}$) mice, we performed western blotting against proteins that we previously observed to be regulated by TC-PTP, notably STAT3 and p38. In naive Mo macrophages, preliminary results indicated that phosphorylated STAT3 (p-STAT3) is upregulated when TC-PTP is lost (**Figure 3.9A**). We observed similar results in M1 macrophages (**Figure 3.9B**). We also appear to see an increase in

phosphorylated p38 (p-p38) in M1 KO BMDMs, but this would need to be further confirmed. Overall, in both Mo and M1 macrophages derived from PTPN2/LysM-Cre mice, we detected a hyperphosphorylation of STAT3 in absence of TC-PTP.

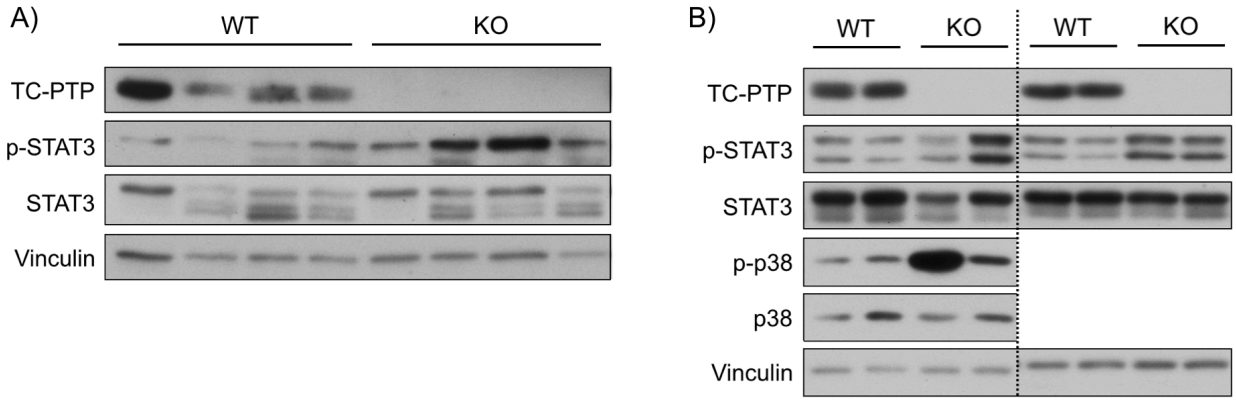

Figure 3.9: Upregulation of phosphorylated STAT3 in Mo and M1 macrophages derived from mice with myeloid-specific deletion of TC-PTP. Total cell lysates (TCL) were prepared from (A) Mo BMDMs and (B) M1 BMDMs derived from PTPN2/LysM-Cre wild-type (WT, wt/wt $Cre^{+/-}$) and knockout (KO, fl/fl $Cre^{+/-}$) mice. Western blotting was performed with 20 µg of lysate. Blots are representative of four mice per genotype. (B) The blots on either side of the dotted line represent different mice from which the TCL was run on separate gels.

3.3.11 Macrophages from TC-PTP myeloid-specific mice have dysregulated pro-inflammatory cytokine production

We next sought to determine if cytokine signaling was altered in TC-PTP-deficient macrophages derived from PTPN2/LysM-Cre mice. As per usual, we harvested WT and knockout KO macrophages from the bone marrow and polarized them towards M1 or M2 macrophages. With the supernatant from these cells, we performed ELISAs to assess the production of the pro-inflammatory cytokines TNF-α, IFN-ɣ and IL-6. While production of TNF-α was independent of TC-PTP expression, we observed an upregulation of IFN-ɣ upon inhibition of TC-PTP (**Figure 3.10**). This is concordant with the phenotype of mice with a systemic deletion of TC-PTP, which have dysregulated cytokine signaling and an

abnormally high expression of IFN-ɣ (28, 102). Of note, the increase in IFN-ɣ production observed in these LysM-Cre TC-PTP knockout macrophages is not as extensive as that observed in mice with a systemic deletion of TC-PTP. Albeit in both instances the macrophages are differentiated and polarized *ex vivo*, it is possible that the transcriptome of these two different macrophage populations may be different from the beginning as a result of cues from the microenvironment and other immune cells before isolating them from the mice. It would be interesting to establish whether the small but significant increase in IFN-ɣ in macrophages derived from mice having a myeloid-specific deletion of TC-PTP is functionally relevant. To do so, infections could be done *in vivo* to determine the physiological role of these macrophages in producing IFN-ɣ and regulating the immune response, particularly the pro-inflammatory response. On the other hand, we noted that TC-PTP KO BMDMs produced about half the amount of IL-6 as their WT counterparts (**Figure 3.10**). This appears to be in contradiction with the levels of the pro-inflammatory cytokine IFN-ɣ. However, various studies have reported that although IL-6 is generally thought of as a pro-inflammatory cytokine, it also exerts anti-inflammatory effects. More specifically, it has been shown to promote polarization of macrophages towards the M2 anti-inflammatory phenotype (248-250). As such, since macrophages lacking TC-PTP seem to be more apt at polarizing towards M1 macrophages and produce more IFN-ɣ, they may somehow downregulate the expression of IL-6 to ensure that the cells do not switch to a more M2 anti-inflammatory population. This needs to be further investigated.

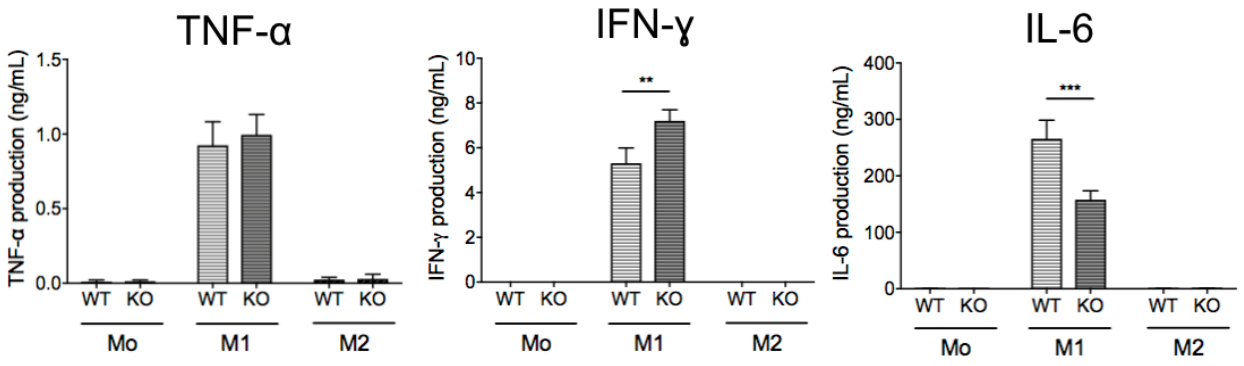

Figure 3.10: Dysregulated cytokine production in LysM-Cre TC-PTP-deficient macrophages. Macrophages were harvested from the bone marrow of LysM-Cre myeloid-specific TC-PTP wild-type (WT) and knockout (KO) mice. Naive BMDMs (Mo) were used, as well as macrophages polarized towards M1 (IFN-ɣ + LPS) and M2 (IL-4) inflammatory phenotypes for 24 hours. Supernatant was collected and ELISA was performed to quantify the expression of the cytokines TNF-α, IFN-ɣ and IL-6 by these macrophages. Data represent mean ± SEM of four mice performed in duplicate. Data were normalized to cell number and analyzed using a 2way ANOVA with a Tukey post-test ** $p < 0.01$; *** $p < 0.005$.

3.3.12 Comparison of systemic and myeloid-specific deletion of TC-PTP *in vivo* using Th1 and Th2 mouse infection models

By using two different mouse models to study the role of TC-PTP, a systemic model and a myeloid-specific model, we aimed to compare the phenotype of the mice as well as their cytokine production and downstream signaling. The experiments conducted thus far have been *ex vivo*. However, we aim to further elucidate whether the absence of TC-PTP solely in myeloid cells would be sufficient to drive inflammation that would lead to an overt phenotype and favour M1 macrophage polarization. To do so, we will be using both systemic and myeloid-specific TC-PTP mouse models and induce two different types of infections that will lead to different immune responses. The first model will require injection of the bacteria *Salmonella typhimurium* to induce a systemic Th1 immune response (**Figure 3.11**). This model is known to produce almost exclusively M1 macrophages due to the host response to the pathogen. At the time of sacrifice, cells will

be collected from the spleen and flow cytometry will be performed to determine the inflammatory phenotype of the macrophages elicited by the immune response. Most importantly, we will want to observe whether there will be a difference in the polarization of these cells *in vivo* in absence of TC-PTP. Pathogen burden will also be assayed from sections of the liver.

The second mouse model will utilize the nematode *Heligmosomoides polygyrus* to induce a Th2 immune response localized to the intestine. This immune response will favour polarization of anti-inflammatory M2 macrophages (**Figure 3.11**). At the time of sacrifice, cells will be harvested from the peritoneal cavity of these mice and flow cytometry performed similarly to the Th1 model. Cytokine production and expression of polarization genes will also be assayed with macrophages from these two models. We expect to see no difference in polarization status in the Th2 infection model. However, based on our previous data, for the Th1 model we expect to observe an increase in the M1 population in TC-PTP-deficient mice, and we predict that this effect will be more pronounced in the systemic mouse model than in the myeloid-specific model. The intriguing question for us is whether myeloid-specific deletion of TC-PTP will be sufficient to drive inflammation and protect the mice from infection.

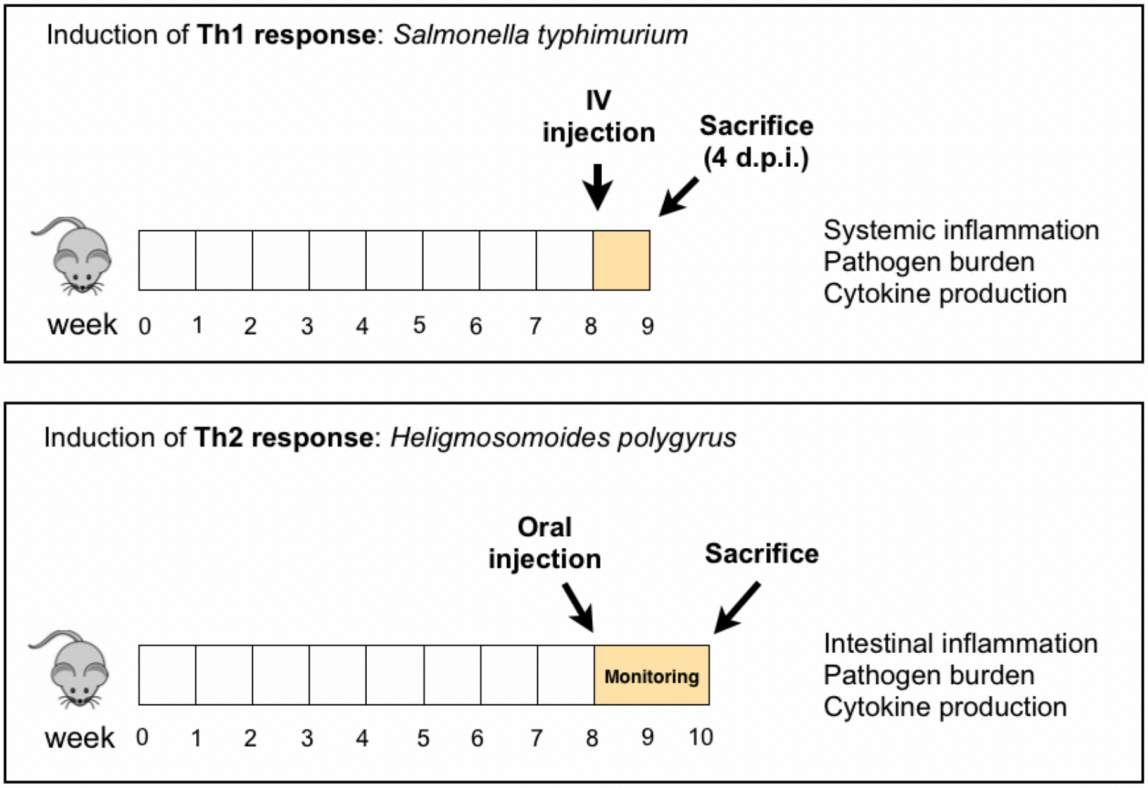

Figure 3.11: Th1 and Th2 *in vivo* infection models. A Th1 infection model using the bacteria *Salmonella typhimurium* and a Th2 model using the nematode *Heligmosomoides polygyrus* will be conducted to induce classical (M1) and alternative (M2) polarization of macrophages, respectively, using both the TC-PTP systemic and LysM-Cre myeloid-specific mouse models. Cells will be harvested at end-point and macrophage polarization will be analyzed by flow cytometry and qRT-PCR.

3.4 Discussion

We have observed in previous studies that the systemic deletion of TC-PTP in mice leads to an aberrant upregulation of pro-inflammatory cytokine signaling, portrayed by increase in IFN-ɣ, TNF-α and iNOS production, that causes severe inflammation and ultimately death (77, 92). We have previously shown that TC-PTP negatively regulates macrophage development and differentiation through the dephosphorylation of the CSF-1R receptor. Consequently, tissues from the spleen of TC-PTP knockout mice have an increased infiltration of F4/80^{+} macrophages (85, 206). We have thus established that

TC-PTP is a negative regulator of pro-inflammatory cytokine signaling as well as macrophage development and differentiation. However, the inflammatory phenotype of macrophages derived from these mice has not yet been studied.

In this study, we have demonstrated that TC-PTP negatively regulates the polarization of macrophages towards the M1 pro-inflammatory phenotype. Systemic deletion of TC-PTP in mice leads to upregulation of M1 markers and genes in macrophages, indicating that absence of TC-PTP renders the macrophages more apt at being polarized towards the pro-inflammatory phenotype. Furthermore, we have established by RNA-Seq that TC-PTP-deficient M1 macrophages have a dysregulated transcriptome and an over-representation of pathways involved in the immune response, macrophage function and metabolism, specifically mitochondrial respiration. When determining the genes that were most downregulated in KO macrophages, we found that these were the same whether the analysis compared them to WT or HET BMDMs. However, what we found intriguing was that within the Mo, M1 and M2 macrophage populations, we were unable to compile a list of genes consistently upregulated in KO macrophages that was comparable between WT and HET macrophages. We were baffled by the significance of this data, if there was any. Possibly, the fact that we could not compose a list of genes that were similarly downregulated in WT and HET BMDMs compared to KO BMDMs implies that the expression of TC-PTP in WT and HET macrophages allows for the tight regulation of genes involved in distinct processes, from immune responses to response to extracellular stimuli to antiviral responses. Deletion of one copy of TC-PTP could be sufficient to alter the transcriptome in an important manner. Perhaps then, when TC-PTP is inhibited, there is an important dysregulation of a plethora

of pathways that is heavily dependent on the extent of its inhibition. In depth studies will need to be performed to identify whether the degree of TC-PTP depletion in mice and specifically in macrophages regulates their immune response and, if so, whether macrophage functions are affected. Nonetheless, our group has already shown in another study that this may be the case, since we observed that TC-PTP heterozygous mice were more susceptible to DSS-induced colitis than their wild-type counterparts (205). This emphasizes the idea that hemi-deficiency of this phosphatase could be sufficient to promote transcriptional changes leading to altered pro-inflammatory responses.

Another group has recently demonstrated using the PTPN2/LysM-Cre model that myeloid-specific deletion of TC-PTP in mice aggravated intestinal inflammation as induced by DSS, but actually protected them from colitis-associated tumour formation. They proposed that these were the consequence of upregulated IL-1β production resulting from increased inflammasome activity (240). However, they did not delve into the phenotype of macrophages and their associated functions. We were interested in determining the inflammatory status of macrophages derived from these PTPN2/ LysM-Cre mice to study their plasticity and immune response resulting from the absence of TC-PTP. Our data suggest that macrophages derived from these mice are more apt to be polarized towards the M1 phenotype in absence of TC-PTP, similarly to the systemic mouse model but to a lower extent. This was accompanied by an increase in IFN-ɣ production but a two-fold decrease in IL-6 production. Considering that IL-6 can act as an anti-inflammatory cytokine that can favour M2 macrophage polarization, it is possible that TC-PTP deficiency in macrophages leads to a reprogramming of their transcriptome to upregulate the IFN-ɣ/STAT1 signaling pathway while downregulating the IL-6/STAT3

pathway to favour a pro-inflammatory microenvironment. Previous reports have demonstrated that induction of M1 macrophage polarization using IFN-ɣ leads to activation of STAT1 through the interferon gamma receptor (IFNGR)-JAK1/JAK2-STAT1 signaling axis (251-253). On the other hand, IL-4 stimulation used to induce M2 macrophage polarization can promote STAT3 activation through the IL-4R-JAK1/JAK3-STAT3 signaling pathway (254, 255). We have shown in PTPN2/LysM-Cre mice that TC-PTP inhibition favours STAT3 hyperphosphorylation. STAT1 is a known substrate of TC-PTP (89), so it is likely that inhibition of TC-PTP would also lead to increased STAT1 activation in macrophages, albeit this will have to be verified. It will be important to investigate which pathways are upregulated in M1 and M2 macrophages to predict their immune response. As such, we propose that inhibition of TC-PTP in macrophages leads to hyperactivation of STAT1 and STAT3, which then dimerize, translocate to the nucleus and induce transcription of various pro-inflammatory and metabolic genes (**Figure 3.12**). STAT1 typically induces the expression of pro-inflammatory genes, while STAT3 as activated by the IL-4R will favour transcription of anti-inflammatory genes (235, 256-259). In the context of colorectal cancer, specifically colitis-associated colorectal cancer (CAC), loss of TC-PTP may promote a pro-inflammatory microenvironment that will induce macrophages to polarize towards the M1 subset and favour immune surveillance and tumor regression. This must be studied in depth to understand if there are interesting avenues regarding inhibition of TC-PTP in immunotherapies to treat CRC and CAC.

Considering that a plethora of JAK and STAT molecules exist within the same cell and that these can be activated by different cytokines, there must be mechanisms in place to ensure proper regulation of these various JAK/STAT pathways. In macrophages, we

believe that TC-PTP may directly and indirectly ensure the proper signaling of these pathways. As we know from the systemic deletion of TC-PTP in mice, they have dysregulated pro-inflammatory cytokine production, including IFN-ɣ, TNF-α and iNOS. Given that expression of the IFN-ɣ receptor can be induced by other pro-inflammatory cytokines (260), our model suggests that in TC-PTP-deficient mice, the upregulation of these pro-inflammatory cytokines could in turn further induce the expression of the IFNGR and increase its activity in comparison to other pathways. This would favour STAT1 activation over STAT3, leading to the predominant expression of pro-inflammatory genes upon translocation of STAT1 to the nucleus. These cytokines would favour a pro-inflammatory microenvironment, which would potentiate M1 macrophage polarization (**Figure 3.12**). In addition, other immune cells in the microenvironment, such as T cells, could be activated by these cytokines and also produce them upon their activation, leading to a synergistic effect for macrophage activation and polarization as well as activation of other immune cells.

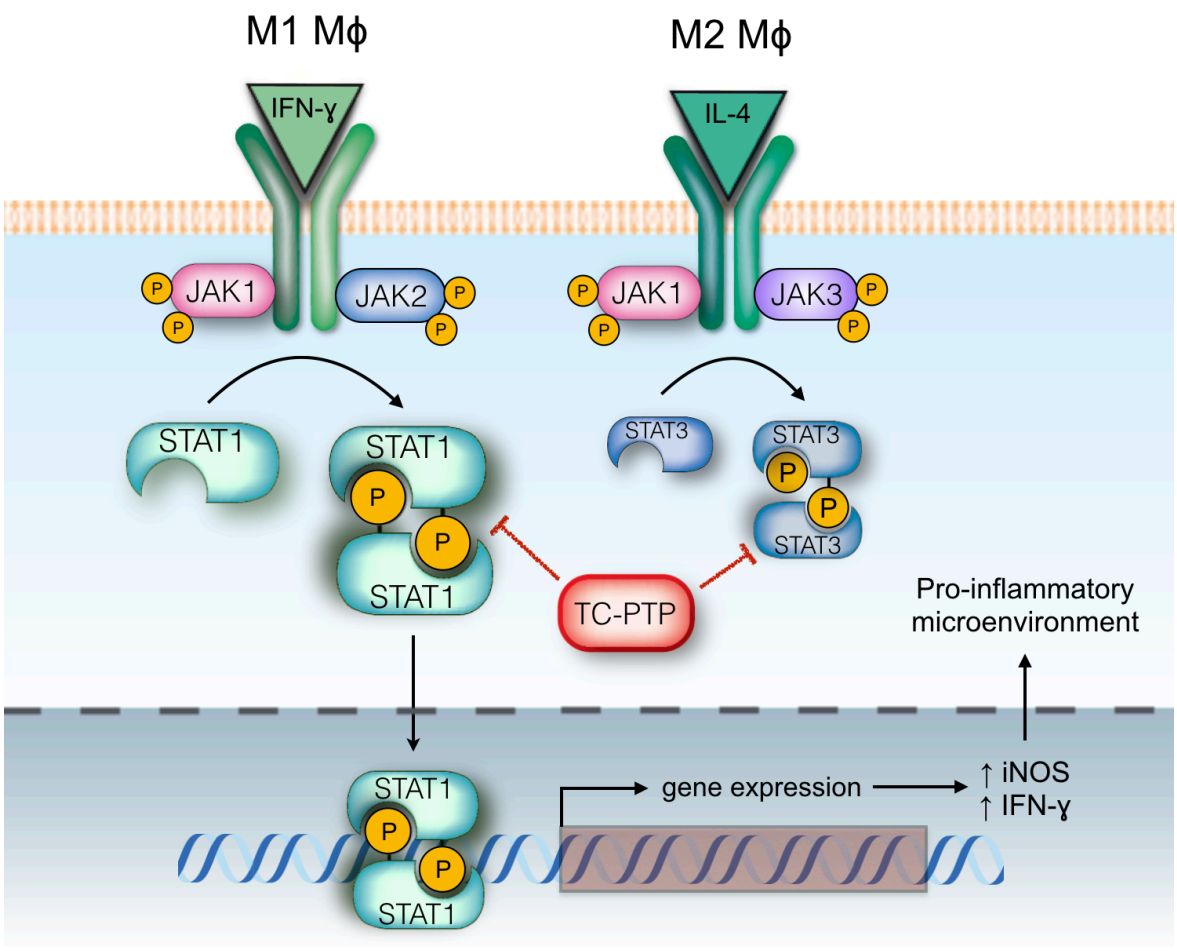

Figure 3.12: Proposed model. In macrophages, inhibition of TC-PTP leads to hyperactivation of STAT3. Given that STAT1 is a known substrate of TC-PTP, it could also be hyperphosphorylated in its absence. We then propose that in absence of TC-PTP, STAT1 and STAT3 are hyperphosphorylated, dimerize and translocate to the nucleus to induce transcription. However, we believe that the IFNGR/JAK/STAT1 signaling pathway may be upregulated in TC-PTP-deficient macrophages due to the upregulation of pro-inflammatory cytokines in these mice. The predominant activation of STAT1 would promote induction of pro-inflammatory genes that would then lead to further cytokine production and result in a pro-inflammatory microenvironment, further exacerbating the effect on macrophages and other immune cells.

Of note, STAT6 can also be activated by the IL-4R following IL-4 cytokine binding (261, 262), so it will be important to determine whether TC-PTP also affects STAT6 phosphorylation in macrophages, particularly since STAT6 has been shown to be its substrate (91).

Interestingly, STAT1 and STAT3 seem to be necessary for ROS-induced mitochondrial respiration and further ROS production (149, 154, 263, 264). Given that we observed in our RNA-Seq M1 macrophage dataset an upregulation of metabolic pathways, specifically those involved in mitochondrial respiration, the upregulation of STAT1 and STAT3 signaling pathways could favour NO and ROS production in TC-PTP-deficient cells, eventually leading to aberrant use of mitochondria by these macrophages. Moreover, STAT3 in the mitochondria has been shown to act as a metabolic sensor through regulation of the electron transport chain in the mitochondria (149). Considering that chronic inflammation can induce metabolic reprogramming (100) and that TC-PTP-deficient mice succumb to severe inflammation, this would be an important avenue to pursue in macrophages.

In the context of cancer and for the purpose of improving immunotherapy, it is essential to establish regulators of macrophage polarization that would favour a pro-inflammatory and anti-tumour microenvironment. Different reports have shown that converting M2 or tumor-promoting macrophages (TAMs) to the pro-inflammatory and anti-tumor M1 macrophage subset leads to tumor regression in different types of cancer, including breast and colorectal cancer (265-267). TAMs are heterogenous macrophages in the tumour microenvironment that are driven by cues from growing cancer cells, and they promote tumour growth and metastasis by inhibiting anti-tumour activity, producing anti-inflammatory cytokines such as IL-10 and TGF-β, and even by secreting factors that promote angiogenesis (268). Although distinct, their phenotype most closely resembles that of M2 anti-inflammatory macrophages. Due to their important role in favouring tumour growth, current immunotherapies involving the reprogramming of TAMs or M2

macrophages to M1 pro-inflammatory macrophages are being proposed. A recent study has demonstrated that an antibody directed against MARCO, a scavenger receptor present on a subset of TAMs, can be used to effectively inhibit cancer progression and metastasis by enhancing the effects of checkpoint therapies in both melanoma and colorectal cancer (268). This report highlights interesting and encouraging possibilities for the use of immunotherapy using macrophages to treat cancer. Interestingly, in our RNA-Seq dataset, there was approximately an 11-fold downregulation of *Marco* in the KO M1 macrophages compared to both WT and HET M1 macrophages. Considering that we observed an upregulation of M1 markers and genes in TC-PTP KO macrophages accompanied by a downregulation of a marker expressed in TAMs, it would be interesting to observe the effects of a combined anti-MARCO antibody and TC-PTP inhibitor approach in macrophages as a potential immunotherapy treatment. Possibly, this “dual-switch” system of reprogramming TAMs or M2 macrophages to M1 pro-inflammatory and anti-tumour macrophages may be beneficial for the treatment of certain cancers. This is a study that would be fascinating to pursue.

CHAPTER 4

PTP1B IS A REGULATOR OF THE INTERLEUKIN 10-INDUCED TRANSCRIPTIONAL PROGRAM IN MACROPHAGES

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4.1 Introduction

In the previous chapter, we demonstrated that the non-receptor protein tyrosine phosphatase TC-PTP regulates the polarization and immune response of macrophages. Another small non-receptor protein tyrosine phosphatase is PTP1B. These two PTPs are 80% similar based on the sequence of their catalytic domain and share some common substrates. Like TC-PTP, PTP1B has been shown to regulate CSF-1R receptor signaling as well as macrophage development and activation (61, 206, 269). Due to their similarities in structure, function and substrate specificities, we were interested in studying the role of PTP1B in regulating the immune response of macrophages. More specifically, knowing that post-translational modifications (PTMs) of STAT proteins are critical to ensure the differential expression of STAT target genes and that reversible tyrosine phosphorylation is a major PTM that regulates various JAK/STAT signaling pathways and to their role in regulating inflammation, we sought to determine whether PTP1B could contribute to modulating the specificity of STAT-dependent gene expression.

It is well known that both pro- and anti-inflammatory cytokines activate JAK/STAT pathways, which typically leads to distinct transcriptional programs. For instance, the cytokine IL-10 is one of the primary anti-inflammatory mediators that is required to resolve inflammation triggered by the immune response. Upon binding to its receptor IL-10R, JAK1 and TYK2 are phosphorylated, leading to recruitment, phosphorylation and activation of STAT3. The newly formed STAT3 dimer shuttles to the nucleus where it promotes the transcription and expression of anti-inflammatory genes (270-272) (**Figure 4.1**). On the other hand, IL-6 binding to its receptor IL-6R leads to phosphorylation of JAK1 and JAK2 which then recruit, phosphorylate and activate STAT3. Translocation

of the STAT3 dimer to the nucleus results instead in the induction of pro-inflammatory gene expression (140, 273, 274) (**Figure 4.1**). Considering that the same JAK or STAT molecules can be activated upon binding of various cytokines that may lead to different immune responses, regulatory mechanisms are essential to ensure the specificity of gene targeting, particularly in response to a diverse array of STAT3-activating cytokines.

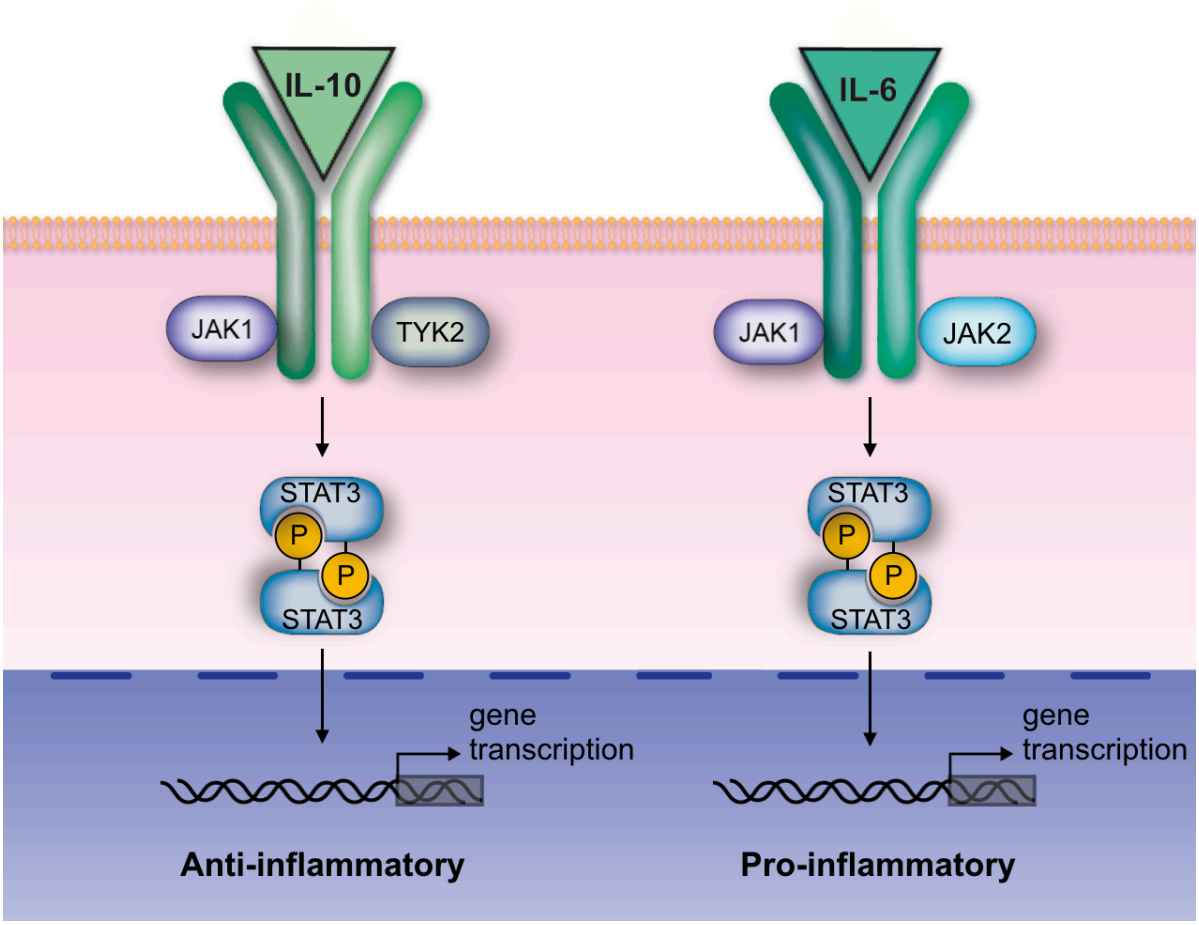

Figure 4.1: Activation of the IL-10 and IL-6 JAK/STAT signaling pathways leads to opposing immune responses. Binding of IL-10 to its receptor IL-10R leads to phosphorylation of JAK1 and TYK2, subsequent phosphorylation of STAT3 and translocation of the STAT3 dimer to the nucleus to induce the expression of anti-inflammatory genes. On the contrary, binding of IL-6 to its receptor IL-6R results in JAK1 and JAK2 phosphorylation, followed by STAT3 phosphorylation and dimerization, and translocation to the nucleus to induce the expression of pro-inflammatory genes. The genes transcribed downstream of JAK/STAT3, notably *SOCS3*, can then negatively regulate the IL-6R signaling pathway and lead to transient STAT3 phosphorylation.

Albeit in both cases STAT3 is activated and translocates to the nucleus, it is the differential phosphorylation of STAT3 that is thought to induce these distinct immune responses. As evidence, the IL-6R induces the expression of SOCS3, which inhibits the IL-6R in a negative feedback loop resulting in only a transient increase in STAT3 phosphorylation (140, 273, 274). The IL-10R, however, is not susceptible to inhibition by SOCS3 and thus the activation of the IL-10/STAT3 pathway leads to prolonged STAT3 phosphorylation and activity. Nonetheless, the molecular mechanisms by which enhanced STAT3 phosphorylation is regulated downstream of the IL-10R to ensure that only genes encoding anti-inflammatory factors are targeted remain undefined. We sought to answer this question.

Through computational analysis of genome-wide STAT3-binding sites in various immune cells, we previously showed that STAT3 has two main modes of binding: 1) a cell type-dependent mode of binding that differs across the four immune cell types tested in our study (macrophages, $CD4^+$ T cells, the mouse pituitary epithelial-like tumour cell line AtT-20 and embryonic stem cells), and 2) a cell type-independent binding mode which is characterized by a set of 35 evolutionary conserved STAT3-binding sites that determine the outcome of STAT activity as well as cell growth (275). Among these 35 cell-independent STAT3 binding mode targets was *Ptpn1* (*PTPN1* in humans*)*, which encodes the protein Ptp1b (PTP1B in humans) (275).

Knowing that TYK2 from the IL-10/STAT3 axis is a substrate of PTP1B, we hypothesized that PTP1B could regulate this pathway by inactivating one of the proteins necessary for the phosphorylation and activation of STAT3 downstream of the receptor. This could explain some immune dysfunctions that are observed in PTP1B-deficient mice,

such as chronic low-grade inflammation (68). In addition, myeloid-specific deletion of PTP1B in mice has been demonstrated to protect against inflammation induced by a high fat diet or LPS due to increased amounts of IL-10 and a hyperphosphorylation of STAT3 (139). However, whether the absence of PTP1B alters the quantitative and qualitative properties of the IL-10-mediated transcriptional profile was not investigated.

In our study, we demonstrated that PTP1B is a negative feedback inhibitor of the IL-10-TYK2-STAT1/3 signaling pathway. Both *in vivo* and by pharmacological means, we showed that in the absence of PTP1B, quantitative and qualitative changes to the IL-10-induced transcriptome do indeed occur. These changes are ascribed to activation of both STAT3 and STAT1. While the hyperphosphorylation of STAT3 corresponded with an increased expression of anti-inflammatory genes, the unexpected increase of STAT1 phosphorylation downstream of the IL-10R was associated with an upregulation of pro-inflammatory genes. In our work, we have showed that PTP1B is a non-redundant inhibitor of the IL-10R/STAT3 signaling and that it is required promote expression of STAT3-dependent anti-inflammatory genes by controlling the threshold of the immune response. We now know that protein tyrosine phosphatases can certainly contribute to regulating STAT-dependent gene targets.

4.2 Materials and Methods

4.2.1 Mice

The generation of *Ptpn1*$^{-/-}$ gene-targeted mice (also known as PTP1B$^{-/-}$) has been described previously (68). Heterozygous breeding pairs were used to ensure that wild-type, heterozygous and knockout littermates were obtained. All mice were housed in a specific pathogen-free facility in sterile microisolator caging. Animal protocols were in

accordance with the regulations of the Canadian Council on Animal Care and were approved by the McGill University animal care committee.

4.2.2. Isolation and stimulation of thioglycolate-elicited peritoneal macrophages

PTP1B$^{-/-}$ mice were subjected to intraperitoneal injection of 1 mL of 3% thioglycolate solution for 3-5 days, after which peritoneal macrophages were harvested by peritoneal lavage with 10 mL of PBS. Macrophages were allowed to adhere to cell culture plates overnight, after which non-adherent cells were removed by extensive washing. Recombinant IL-10 (PeproTech) was added at a concentration of 10 or 100 ng/mL, and IL-6 (PeproTech) and LPS (Sigma-Aldrich) were used at 100 ng/mL for the specified time. In experiments where the PTP1B inhibitor was used, cells were pretreated with the inhibitor overnight before the addition of IL-10.

4.2.3 RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction

For peritoneal macrophages, total RNA was extracted using the TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Potential contaminating genomic DNA was degraded with the DNase I RiboPure kit (Thermo Fisher Scientific). RNA was quantified with the NanoDrop 100 Spectrophotometer (Thermo Fisher Scientific) and 1 µg was transcribed to cDNA with the SuperScript III Reverse Transcriptase Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a LightCycler 480 (Roche) with SYBR Green Master Mix (Roche) according to the manufacturer's instructions. *Gapdh* was used as a reference gene to normalize the relative abundance of the genes of interest.

4.2.4 Flow cytometry

Fc receptors on purified peritoneal macrophages were blocked with anti-CD16 antibodies (BD Biosciences). Cell surface antigens were detected with the following antibodies diluted in Dulbecco's PBS supplemented with 2.5% fetal calf serum: phycoerythrin (PE)-Cy5-conjugated anti-F4/80 and PE-conjugated anti-CD11b. For experiments requiring detection of cytokine production by flow cytometry, GolgiStop (BD Biosciences) was added to macrophage cultures 4 hours before cells were harvested. Accumulated intracellular cytokines were detected by fixing and permeabilizing cells with BD Cytofix/Cytoperm (BD Biosciences). All samples were collected with a FACSCalibur instrument (BD Biosciences) and analyzed with FlowJo software (Tree Star). One-way ANOVA with a Tukey post-test was performed on independent experiments detecting IL-4Rα^{HIGH} cells.

4.2.5 Western blotting

Total cell lysates were prepared using mRIPA supplemented with EDTA-free protease inhibitor (Roche) and 2 mM sodium orthovanadate (Sigma-Aldrich). Lysates were resolved by SDS-PAGE and transferred to membranes for western blotting. The antibodies used for western blotting were rabbit primary antibodies used at a 1:1,000 dilution and purchased from Cell Signaling Technology: anti-phospho-STAT3 (Y705), anti-STAT3, anti-phospho-STAT1 (Y701) and anti-STAT1. The mouse anti-calnexin primary antibody was used at a 1:5,000 dilution and was provided by J.J.M. Bergeron (McGill University) and the rabbit anti-PTP1B (ABS40, 1:1,000 dilution) was obtained from Millipore. The secondary antibodies HRP-conjugated goat anti-mouse and goat anti-rabbit immunoglobulins (Jackson Laboratories) were used at a final dilution of

1:5,000. Quantity One Software (Bio-Rad) was used to determine the pixel intensity of the bands. One-way ANOVA with a Tukey post-test was performed on normalized pSTAT3 pixel intensities from independent experiments.

4.2.6 RNA-sequencing and data analysis

RNA from thioglycollate-elicited peritoneal macrophages was isolated with TRIzol and subjected to RNA-Seq on an Illumina HiSeq 2000. Paired-end reads were aligned to the mouse transcriptome (Ensembl v67) with RSEM software v1.1.21 (276) and bowtie software v0.12.7 (277). For a gene to be considered for downstream analysis, we set the threshold to at least 20 mapping sequence tags in at least two biological samples. RNA-seq data were then normalized for GC (guanine-cytosine) content with EDASeq software v1.4.0 (53), and differential expression was determined with edgeR software v3.0.8 (278) with a q value of 0.05. Genes were then further filtered on the basis of at least two-fold increase in expression and were assigned to specific categories depending on their fold change amongst the different genotypes. GO enrichment analysis was performed with PANTHER. Raw data were deposited in Gene Expression Omnibus with accession number GSE49449.

4.3 Results

4.3.1 PTP1B deficiency in macrophages results in increased phosphorylation and transcriptional activity of STAT3 in response to IL-10

In a previous study, we showed that one of the main gene targets of STAT3 is *Ptpn1*, and this independent of cell type (275). To study the contribution of PTP1B in regulating the phosphorylation and transcriptional activity of STAT3, we isolated and purified thioglycollate-elicited peritoneal mouse macrophages derived from PTP1B wild-type ($PTP1B^{+/+}$), heterozygous ($PTP1B^{+/-}$) or knockout ($PTP1B^{-/-}$) mice and stimulated them with IL-10. Within 2 hours of stimulation with IL-10, we started seeing an increase in phosphorylated STAT3 protein abundance that was further upregulated at 4 hours. Whereas there was no difference in STAT3 phosphorylation between $PTP1B^{+/+}$ and $PTP1B^{+/-}$ macrophages, $PTP1B^{-/-}$ macrophages had a 1.6- to 2-fold increase in the abundance of phosphorylated STAT3 (pSTAT3) compared to $PTP1B^{+/+}$ macrophages (**Figure 4.2A**). In a similar manner, we stimulated macrophages with IL-6 for 2 and 4 hours and observed an upregulation of phosphorylated STAT3 beginning at 2 hours and further increasing in abundance at 4 hours. However, there was no difference in pSTAT3 protein abundance in $PTP1B^{-/-}$ macrophages compared to both $PTP1B^{+/+}$ and $PTP1B^{+/-}$ macrophages upon stimulation with IL-6 (**Figure 4.2B**), indicating that PTP1B does not regulate the IL-6/STAT3 signaling pathway in macrophages.

To determine whether the upregulation of pSTAT3 in $PTP1B^{-/-}$ macrophages was sufficient to induce transcriptional changes, we performed qRT-PCR analysis to quantify the gene expression of known IL-10-induced STAT3 target genes. The genes analyzed produce proteins that are implicated in directing the anti-inflammatory response by acting as transcriptional co-repressors (*Bcl3* and *Sbno2*), inhibiting pro-inflammatory cytokine

receptor signaling (*Socs3*) or compromising tumor necrosis factor α (TNF-α) mRNA stability (*Zfp36*). *Bcl3*, *Sbno2*, *Soc3* and *Zfp36* gene expression was upregulated in $PTP1B^{-/-}$ peritoneal macrophages compared to $PTP1B^{+/+}$ macrophages after 2 and 8 hours of stimulation with IL-10 (**Figure 4.2C**). Interestingly, we noted that the expression of these genes reached a plateau at 4 hours of treatment in $PTP1B^{+/+}$ and $PTP1B^{+/-}$ macrophages, but continued to increase in abundance in $PTP1B^{-/-}$ macrophages until 8 hours after stimulation with IL-10 (**Figure 4.2C**). To confirm that PTP1B does not play a role in regulating the IL-6/STAT3 axis in macrophages, we performed qRT-PCR and looked at the same set of STAT3 anti-inflammatory target genes after IL-6 stimulation from 0 to 6 hours. Although there was an increase in the genes analyzed after treatment, particularly in *Stat3* and *Socs3* expression, there was no difference between $PTP1B^{+/+}$ and $PTP1B^{-/-}$ macrophages, indicating that PTP1B does not phosphorylate and activate STAT3 through the IL-6 receptor (**Figure 4.2D**).

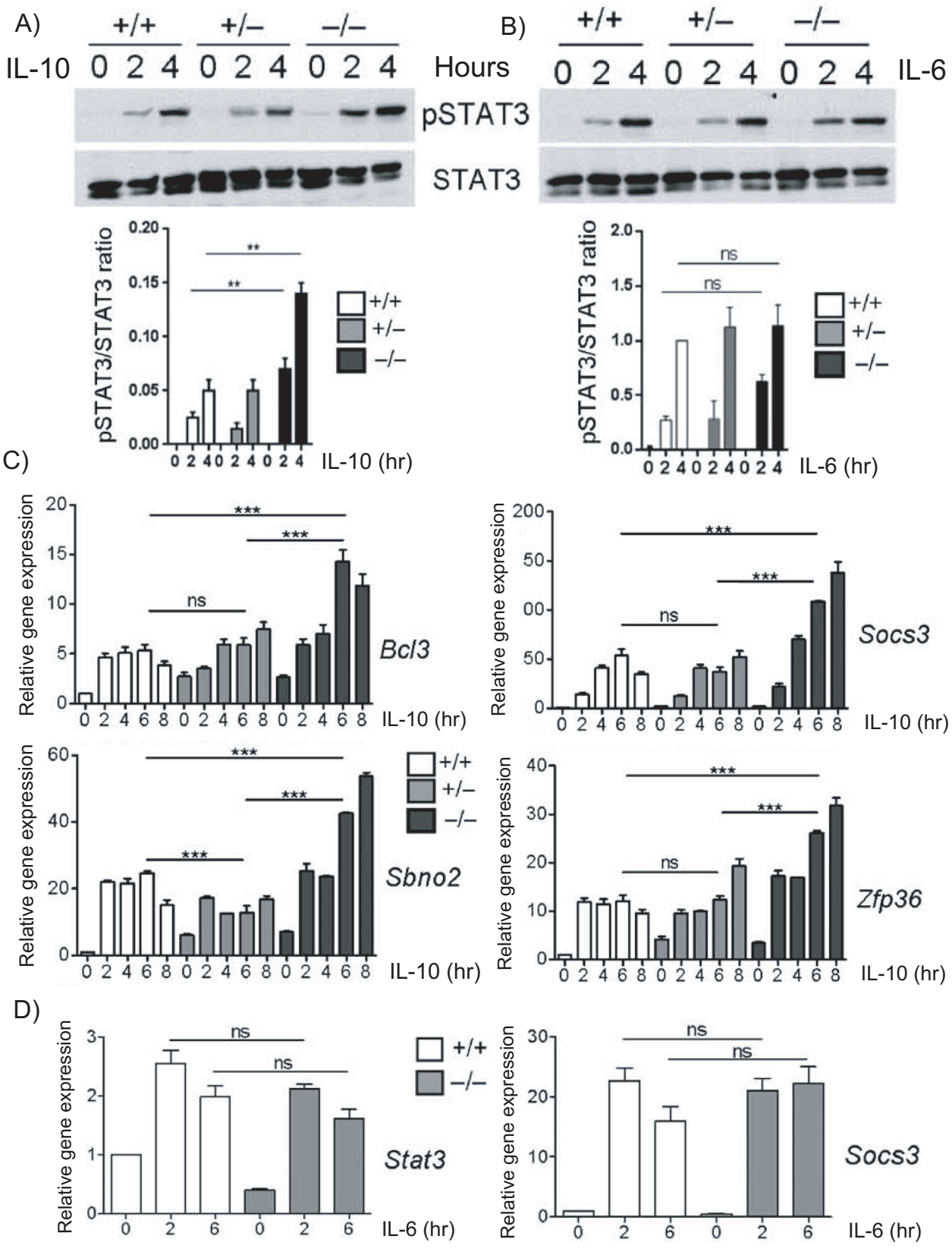
A)
+/+
+/–
–/–
IL-10
0 2 4 0 2 4 0 2 4
Hours
pSTAT3
STAT3
pSTAT3/STAT3 ratio
IL-10 (hr)
B)
IL-6
IL-6 (hr)
C)
Relative gene expression
Bcl3
Socs3
Sbno2
Zfp36
D)
Stat3
Socs3
ns
***
**

Figure 4.2: PTP1B deficiency results in increased phosphorylation and transcriptional activity of STAT3 in response to IL-10, but not to IL-6. Thioglycollate-elicited peritoneal macrophages were harvested from $PTP1B^{+/+}$, $PTP1B^{+/-}$ and $PTP1B^{-/-}$ mice and were stimulated with IL-10 (A and C) or IL-6 (B and D). (A and B) Cells were harvested after 2 or 4 hours of IL-10 (A) or IL-6 (B) stimulation and subjected to western blotting. Densitometry analysis was performed to determine the relative abundance of pSTAT3 relative to total STAT3. Data are means ± SEM of three independent experiments. ** $p < 0.01$. (C and D) qRT-PCR analysis was performed with purified cDNA from these macrophages to quantify transcription levels of several genes upon stimulation with IL-10 (C) or IL-6 (D). Data represent the means ± SEM of one experiment with three technical replicates and is representative of three independent experiments. Samples were normalized to the reference gene *Gapdh*. *** $p < 0.001$; ns: not significant.

4.3.2 Loss of PTP1B results in increased IL-4Rα cell surface abundance upon IL-10 stimulation

Apart from inducing the expression of genes that promote an anti-inflammatory response, IL-10 also primes macrophages to polarize towards the M2 anti-inflammatory phenotype by increasing the cell surface abundance of the IL-4Rα chain (216). With qRT-PCR analysis, we found that IL-10 stimulation of macrophages increased IL-4Rα gene expression by 3-fold in $PTP1B^{-/-}$ cells compared to both $PTP1B^{+/+}$ and $PTP1B^{+/-}$ cells (**Figure 4.3A**). This upregulation of IL-4Rα mRNA level correlated with an increase in the protein abundance on the cell surface. We performed flow cytometry to assess the expression of both IL-4Rα and IL-15Rα in absence or presence of IL-10 in macrophages. In the absence of IL-10, less than 5% of macrophages had a high expression of IL-4Rα at the cell surface ($IL\text{-}4R\alpha^{HIGH}$), independent of the presence or absence of PTP1B (**Figure 4.3B**). Stimulation with IL-10 resulted in about 18% of $PTP1B^{+/+}$ cells to be $IL\text{-}4R\alpha^{HIGH}$, whereas 54% of $PTP1B^{-/-}$ macrophages had a high cell surface abundance of IL-4Rα (**Figure 4.3B**). Hence, there was a three-fold upregulation of $IL\text{-}4R\alpha^{HIGH}$ cells in absence of PTP1B and in response to IL-10. We did not see any difference in the cell

surface expression of IL-15Rα, indicating that IL-10 stimulation of PTP1B-deficient macrophages induces the specific expression of the IL-4Rα chain.

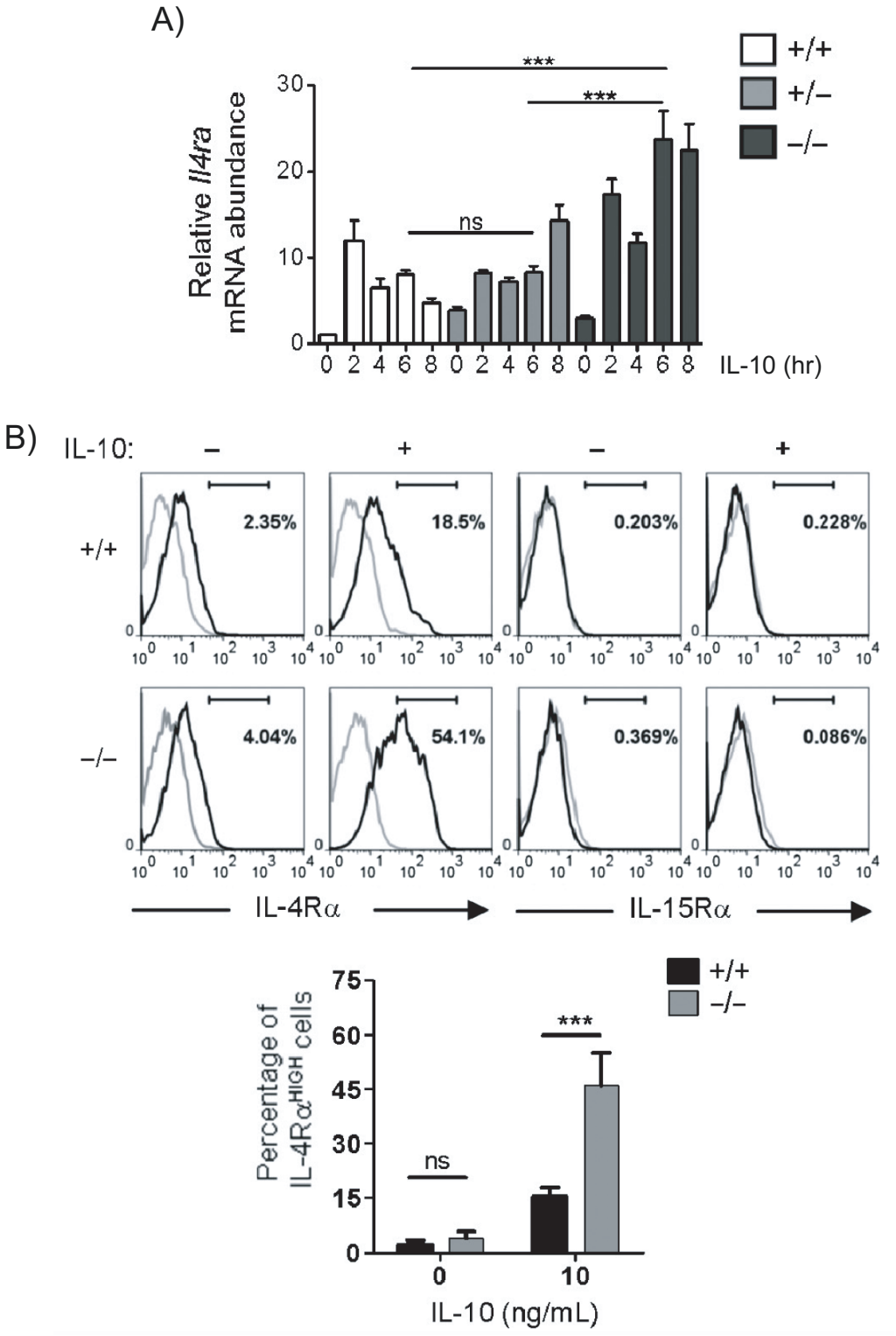

Figure 4.3: Absence of PTP1B leads to an increase in cell surface abundance of IL-4R in response to IL-10. (A) Total RNA was isolated from IL-10-stimulated $PTP1B^{+/+}$, $PTP1B^{+/-}$ and $PTP1B^{-/-}$ peritoneal macrophages and qRT-PCR was performed to determine the relative gene expression of IL-4Rα. Samples were normalized to the reference gene *Gapdh* and represent the means ± SEM of one experiment representative of three independent experiments. *** $p < 0.001$. (B) To determine the cell surface abundance of IL-4Rα and IL-15Rα, macrophages from $PTP1B^{+/+}$ and $PTP1B^{-/-}$ mice were either unstimulated or stimulated with IL-10 overnight before being analyzed by flow cytometry. Histograms are representative of three independent experiments. A bar graph shows the means ± SEM of the percentage of IL-4R high cells ($IL\text{-}4R\alpha^{HIGH}$) from three independent experiments. *** $p < 0.001$.

4.3.3 Pharmacological inhibition of PTP1B validates the IL-10-induced increase of IL-4Rα at the cell observed in PTP1B knockout macrophages

After observing that $PTP1B^{-/-}$ macrophages had three times the number of $IL\text{-}4R\alpha^{HIGH}$ cells compared to $PTP1B^{+/+}$ macrophages after treatment with IL-10, we wondered if we could recapitulate this data using a PTP1B inhibitor. Considering that the macrophages that we harvest are obtained from mice who have a systemic deletion of the protein, inhibiting PTP1B on isolated macrophages would allow us to confirm the cell-intrinsic effect of PTP1B deficiency. We used difluoromethyl phosphonate (DFMP) inhibitor 7-bromo-6 phosphono(difluoromethyl)-3-napthalenonitrile (PTP1Bi) to effectively inhibit PTP1B (279, 280). Of note, PTP1Bi is an order of magnitude more selective for PTP1B than for the highly similar phosphatase TC-PTP (281). We treated peritoneal macrophages from $PTP1B^{+/+}$ mice with PTP1Bi before stimulating them with IL-10 for 2 or 6 hours and we observed an increase in pSTAT3 levels within 2 hours of stimulation and increasing at 6 hours (**Figure 4.4A**). We performed qRT-PCR to determine if the IL-10-induced STAT3 target genes would be upregulated, and indeed we observed an increased gene expression of *Socs3* after 2 and 6 hours of stimulation in presence of PTP1Bi compared to control cells (**Figure 4.4B**). Just like in $PTP1B^{-/-}$ mice, we saw an

increase in the abundance of IL-4Rα at the cell surface in response to IL-10 when depleting PTP1B with an inhibitor compared to untreated cells (**Figure 4.4C**). Pharmacological inhibition of PTP1B allowed us to recapitulate the phenotype that we observed in macrophages harvested from PTP1B$^{-/-}$ mice.

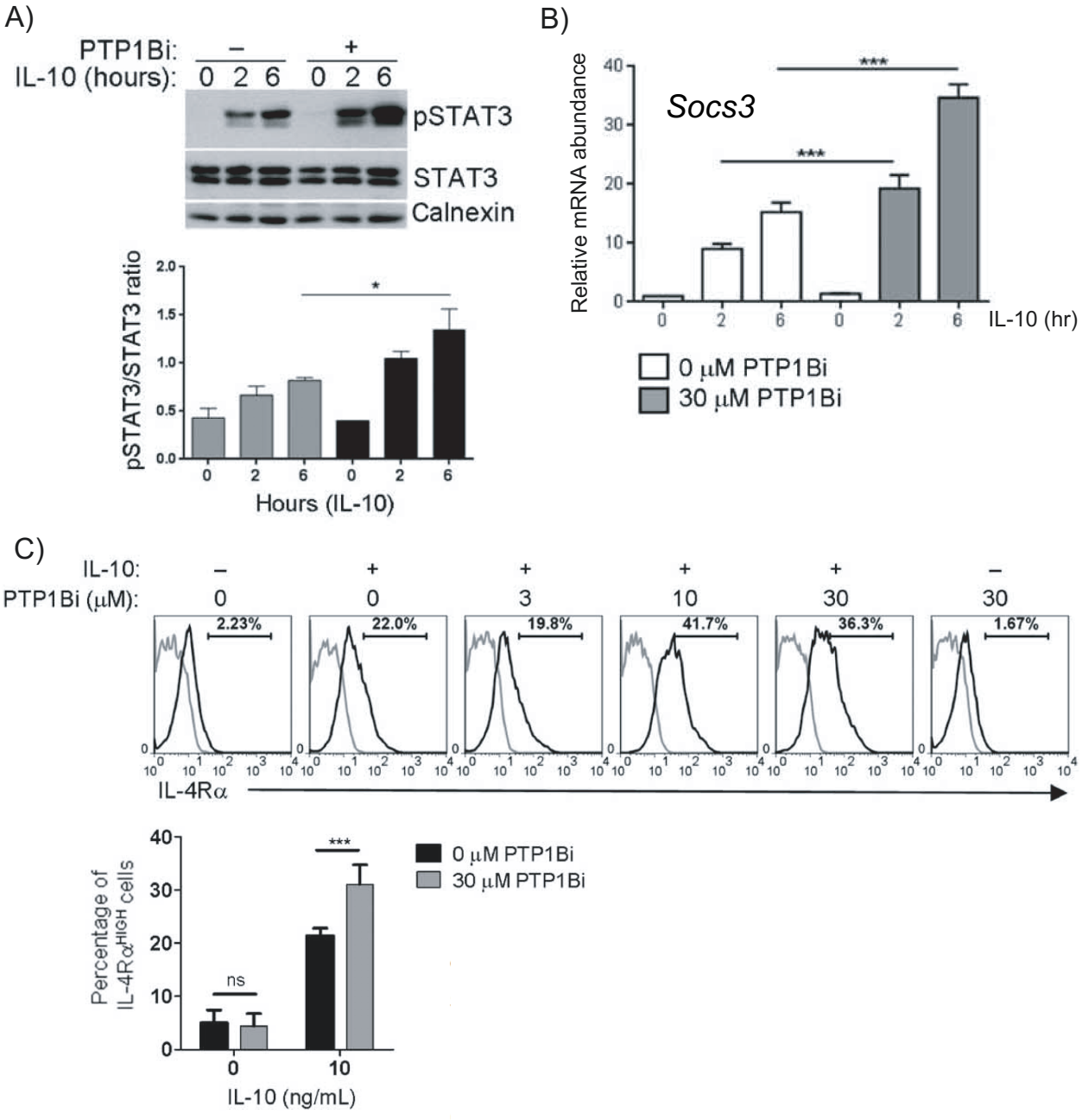

Figure 4.4: Pharmacological inhibition of PTP1B increases IL-10R-dependent STAT3 activation, target gene transcription and IL-4Rα abundance at the cell surface. (A) PTP1B$^{+/+}$ macrophages were pretreated with 30 μM PTP1B inhibitor (PTP1Bi) or vehicle control and were then stimulated with IL-10 for 2 or 6 hours. Total cell lysates were analyzed by western blotting with antibodies against pSTAT3, STAT3 and calnexin. Bar graph shows the relative abundance of pSTAT3 normalized to total STAT3 in non-treated (gray) and PTP1Bi-treated (black) macrophages. Data represent means ± SEM of four independent experiments. * $p < 0.05$. (B) Total RNA was purified from unstimulated and IL-10-stimulated PTP1B$^{+/+}$ macrophages with or without pretreatment with 30 μM PTP1Bi. We performed qRT-PCR to determine *Socs3* gene expression in these macrophages. Samples are normalized to the reference gene *Gapdh* and data are means ± SEM and is representative of three independent experiments. *** $p < 0.001$. (C) PTP1B$^{+/+}$ macrophages were treated overnight with the indicated concentrations of PTP1Bi in the presence of absence of IL-10. Samples were then analyzed by flow cytometry to determine the percentage of IL-4Rα^{HIGH} cells. Bar graph shows the percentage of IL-4Rα^{HIGH} cells. Data represent means ± SEM of three independent experiments. *** $p < 0.001$.

4.3.4 Critical quantitative and qualitative changes to the IL-10-induced transcriptome in PTP1B-deficient macrophages

Albeit our findings demonstrated that PTP1B plays a critical role in inhibiting the activity of STAT3 downstream of the IL-10R, we wanted to further study whether the loss of PTP1B would affect the transcriptome of macrophages in response to IL-10. To answer this question, we performed RNA-Seq to identify quantitative and qualitative changes in the IL-10-induced transcriptional program by isolating RNA from unstimulated and IL-10-stimulated peritoneal macrophages derived from PTP1B$^{+/+}$ and PTP1B$^{-/-}$ mice. Given that sequencing was performed on macrophages stimulated with IL-10 for 4 hours, the modulation of gene expression varied from gene to gene, which probably reflects their different transcriptional kinetics in response to IL-10. After analysis, we found two main gene sets that were changed in our conditions. The first reflects a normal anti-inflammatory response in which gene expression was induced in both PTP1B$^{+/+}$ and PTP1B$^{-/-}$ macrophages upon IL-10 stimulation, but to a greater extent in the latter. Unexpectedly, we found that the second subset contained 42 genes that were only

upregulated in PTP1B$^{-/-}$ macrophages upon IL-10 stimulation and these were not anti-inflammatory factors (**Figure 4.5**). Upon further characterization, we found that this second gene set consisted of pro-inflammatory genes, and their expression seem to correlate with the activation of STAT1. By comparing the expression of genes increased in response to IL-10 to unstimulated cells, we found 78 genes to be upregulated in both PTP1B$^{+/+}$ and PTP1B$^{-/-}$ macrophages, 10 genes were upregulated only in PTP1B$^{+/+}$ macrophages and 42 genes were specifically upregulated in PTP1B$^{-/-}$ macrophages (**Figure 4.5**). Evidently, the loss of PTP1B in macrophages results in quantitative as well as qualitative changes in IL-10-induced transcriptome.

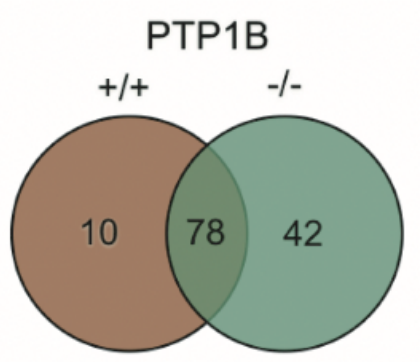

Figure 4.5: Loss of PTP1B in macrophages leads to quantitative and qualitative changes to the IL-10-induced transcriptome. PTP1B$^{+/+}$ and PTP1B$^{-/-}$ macrophages were treated with IL-10 for 4 hours and analyzed by RNA-Seq to determine the effect of the loss of PTP1B on the IL-10-induced transcriptome. Representation of the number of genes whose expression is specifically increased in PTP1B$^{+/+}$ and PTP1B$^{-/-}$ macrophages in response to IL-10, also indicating the overlap of 78 genes that are increased in both populations in response to IL-10.

4.3.5 IL-10 aberrantly induces the transcription of a pro-inflammatory gene set in absence of PTP1B

Following the observation that 42 genes were specifically upregulated in PTP1B$^{-/-}$ macrophages in response to IL-10, we performed gene ontology (GO) enrichment analysis to identify potential pathways or biological processes specific to PTP1B-deficient macrophages. GO enrichment analysis allowed us to identify the top biological processes represented in these PTP1B$^{-/-}$ macrophages: response to IFN-ɣ, immune response,

immune system process and response to stimulus (**Figure 4.6A**). Interestingly, most of the genes represented in these pathways are pro-inflammatory in nature. By comparing the average counts per million (cpm) of transcripts belonging to these top GO terms, including the IFN-inducible (IFI) proteins, IFN-regulatory factors (IRFs) and small IFN-inducible guanylate-binding proteins (GBPs), we found that these had an overall increase in cpm in PTP1B-deficient macrophages, both in response to IL-10 and in basal conditions. After observing that a subset of pro-inflammatory genes was upregulated in IL-10-stimulated PTP1B$^{-/-}$ macrophages, we looked at the phosphorylation of STAT1 in these macrophages after stimulation with various concentrations of IL-10 for 4 hours. We observed a hyperphosphorylation of STAT1 in PTP1B-deficient macrophages in response to IL-10, indicating that absence of PTP1B leads to an aberrant downstream activation of STAT1 (**Figure 4.6B**).

We next wanted to confirm whether the pro-inflammatory genes induced by STAT1 activation would indeed be upregulated in PTP1B-deficient macrophages. Although not all the genes encoding IRFs were upregulated, we found that almost all IFI and GBP genes transcripts verified had an increase in abundance, as was determined by RNA-Seq. We performed qRT-PCR with PTP1B$^{+/+}$ and PTP1B$^{-/-}$ macrophages stimulated or not with IL-10 and confirmed that some of these target genes, such as *Ifi47*, *Oas2*, *Gbp2* and *Gbp6*, were increased in knockout macrophages in response to IL-10 (**Figure 4.6C**). RNA-Seq as well as validation by qRT-PCR of key gene targets confirmed that there is indeed an erroneous upregulation of a pro-inflammatory gene program in PTP1B$^{-/-}$ peritoneal macrophages in response to IL-10 as a consequence of STAT1 activation.

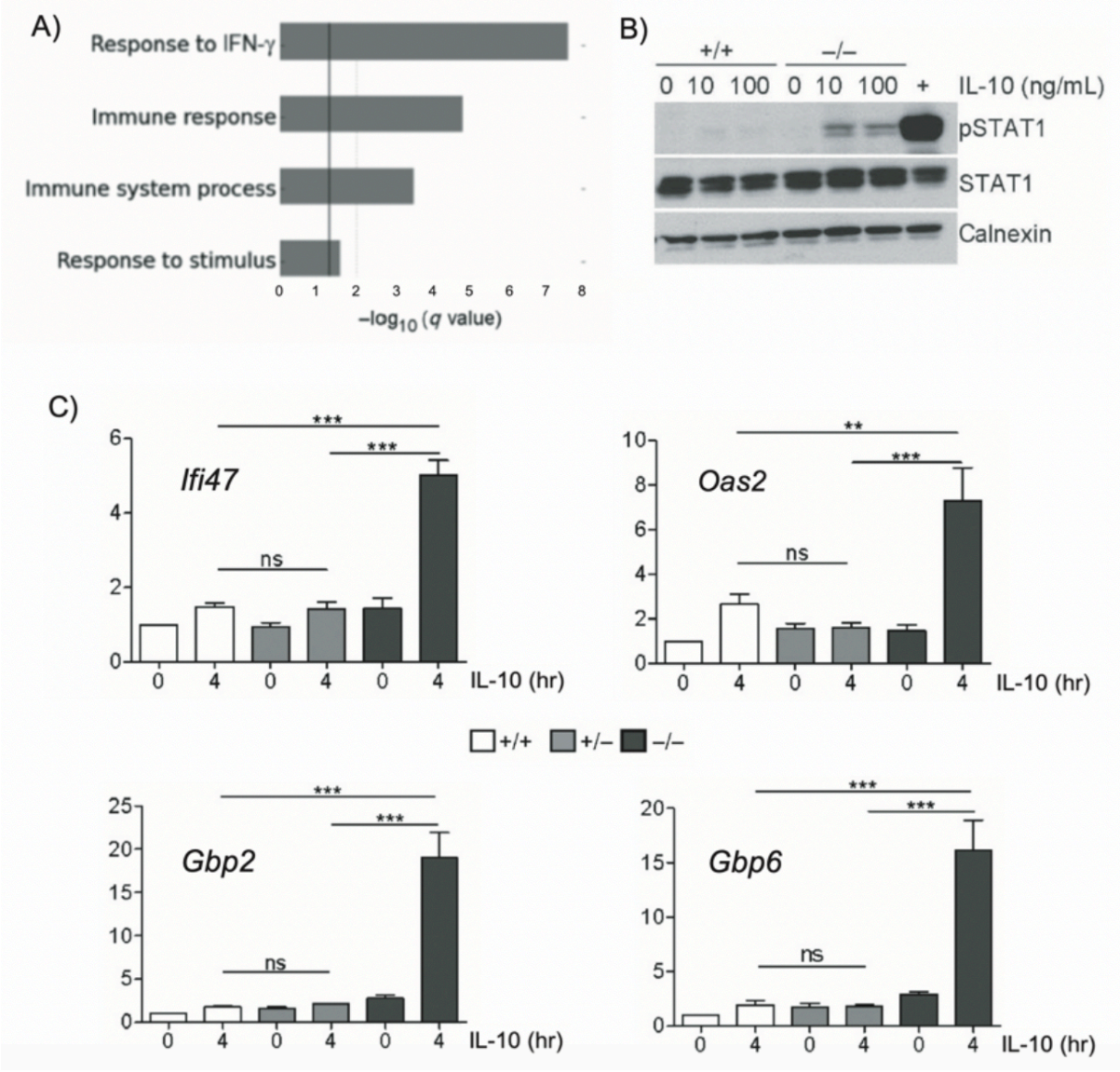

Figure 4.6: IL-10 aberrantly induces the activation of STAT1 and leads to transcription of pro-inflammatory in PTP1B-deficient macrophages. (A) GO terms that describe genes of the category "Biological Process" whose expression in IL-10-stimulated PTP1B$^{-/-}$ macrophages were increased compared to PTP1B$^{+/+}$ macrophages. (B) PTP1B$^{+/+}$ and PTP1B$^{-/-}$ macrophages were stimulated with different concentrations of IL-10 for 4 hours. Total cell lysates were analyzed by western blotting for pSTAT1, STAT1 and calnexin. Western blots are representative of three independent experiments. IFN-ɣ-stimulated cells were used as a positive (+) control for pSTAT1. (C) To validate the pro-inflammatory gene set that was upregulated in PTP1B$^{-/-}$ macrophages in response to IL-10 as observed in our RNA-Seq dataset, qRT-PCR was performed to determine their gene expression. *Ifi47*, *Oas2*, *Gbp2* and *Gbp6* were measured and their gene expression normalized to the reference gene *Gapdh*. Data represent the means ± SEM of one experiment representative of three independent experiments. ** $p < 0.01$, *** $p < 0.001$.

4.3.6 Proposed model of PTP1B regulation of the IL-10/STAT3 pathway in macrophages

In presence of PTP1B and upon IL-10 stimulation, some STAT3 is phosphorylated, leading to the increase in the expression of anti-inflammatory genes such as *Bcl3*, *Nfil3*, *Etv3*, *Sbno2*, *IL4ra* and *Zfp36*. In instances where PTP1B is deficient, STAT3 is hyperphosphorylated since PTP1B is not present to dephosphorylate it, and this leads to an upregulation of the aforementioned STAT3 anti-inflammatory gene targets. However, absence of PTP1B also leads to increased phosphorylation of STAT1, which results in an unexpected increase of a pro-inflammatory gene set including *Ifi47*, *Oas2*, *Gbp2*, *Gbp6* and many others, thus shifting the balance of anti- to pro-inflammatory genes being transcribed (**Figure 4.7**). Although pro-inflammatory genes are upregulated in PTP1B-deficient macrophages, we suspect that the microenvironment would remain predominantly anti-inflammatory in nature because of the much higher response and activity of the anti-inflammatory STAT3 gene targets. Furthermore, deregulation of inflammatory signals, particularly STAT3, have been shown to promote immune suppression (282, 283). As such, hyperactivation of STAT3 resulting from the inhibition of PTP1B would likely promote an immunosuppressive microenvironment in macrophages.

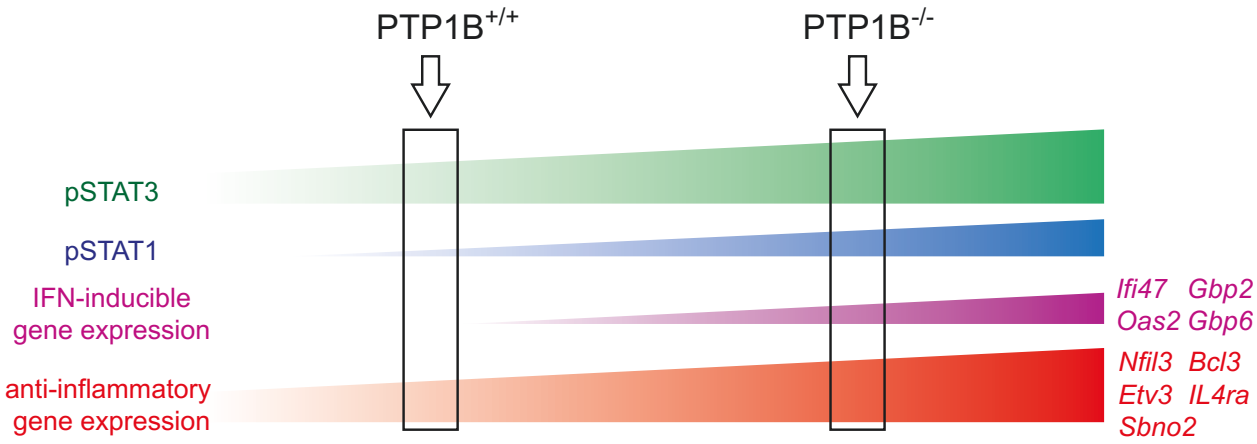

Figure 4.7: Summary of IL-10-induced changes in the transcriptome of PTP1B-deficient macrophages. Absence of PTP1B in macrophages leads to upregulation of pSTAT3 in response to IL-10, resulting in an increase in several genes involved in the anti-inflammatory response. Interestingly, we also observed a hyperactivation of pSTAT1 in these PTP1B-deficient macrophages upon IL-10 stimulation, leading to an increase in pro-inflammatory gene targets not observed in PTP1B$^{+/+}$ macrophages. Figure from Noriko Uetani.

4.4 Discussion

It has long been known that during the immune response, IL-10 has a critical and non-redundant role in controlling inflammation. It is a potent suppressor of pro-inflammatory cytokine production in macrophages and other immune cells in the context of inflammation of the colon, thereby dampening the immune response (284, 285), it mediates anti-viral (286, 287) and parasitic (288) immune suppression in T cells, and is essential in combatting a lethal inflammatory response in mice exposed to malarial parasites (289). IL-10R signaling can be controlled by the availability of the IL-10 cytokine (290) and the degradation of the cell surface IL-10R1 after stimulation with IL-10 and subsequent decrease in STAT3 activation (291). Although the mechanisms by which IL-10R activates STAT3 have been studied extensively, the downstream regulatory

mechanisms by which the extent of STAT3 phosphorylation and specificity is regulated in response to IL-10 is only partially understood. We have described this previously uncharacterized mechanism in our study.

Our findings demonstrate that PTP1B is an inducible inhibitor of the IL-10/TYK2/STAT3 signaling axis since PTP1B-deficient macrophages have a prolonged TYK2 phosphorylation as well as hyperphosphorylated STAT3 in response to IL-10. This leads to a specific upregulation of STAT3 target genes and a more efficient suppression of macrophage activation. Interestingly, although several PTPs such as TC-PTP (90), SHP-1 (292, 293), SHP-2 (294), PTPRE (295) and PTPRT (296) regulate the JAK/STAT3 pathway, it appears that the absence of PTP1B cannot be compensated by these PTPs. Since the specificity and activity of PTPs are highly dependent on their subcellular localization, it is likely that the non-redundancy of PTP1B is explained by its unique localization. While SHP1, SHP2 and the predominant isoform of TC-PTP are localized to the nucleus (297-301), and the receptors PTPRE and PTPRT are found at the cell surface, PTP1B is mostly found in the cytoplasm. The different subcellular localizations of these PTPs may explain their various functions and kinetics upon IL-10 stimulation (302). Due to its cytoplasmic distribution, PTP1B may be more essential in regulating early, membrane-proximal events such as TYK2 activation. On the other hand, TC-PTP or other PTPs may play a greater role in the nucleus downstream of STAT3 activation. The cell type and cellular context are also important. For instance, PTPRT targets STAT3 in colorectal cancer (296), but not in keratinocytes (303). More work will be required to determine how different PTPs coordinate their response within one cell and why particular PTPs are more or less important in specific cell lineages.

With regards to PTP1B, we know that different inflammatory cues upregulate PTP1B protein abundance, so increased PTP1B may in fact reduce any IL-10-dependent anti-inflammatory responses by inhibiting signaling downstream of the IL-10R (269, 304, 305). It has also been demonstrated that decreased PTP1B abundance results in an upregulation of the number of myeloid suppressor cells derived from bone marrow cells and that this provides protection against chronic inflammation and colitis (306). These various studies highlight the role of PTP1B in controlling the anti-inflammatory response in difference cell types. Nonetheless, PTP1B does not only function as a mediator of the anti-inflammatory response. It can also negatively regulate pro-inflammatory responses, as can be observed in PTP1B-deficient mice since these have exacerbated inflammation and increased leukocyte trafficking during allergic responses (307). In addition, T cells derived from PTP1B-deficient mice produce more of the pro-inflammatory cytokine IFN-ɣ in response to mitogenic stimulants (206). These studies demonstrate that lack of PTP1B in immune cells can also favour a pro-inflammatory response, highlighting its dual role in regulating both anti- and pro-inflammatory responses.

PTP1B inhibitors are being tested for the treatment of obesity and type II diabetes. Since both of these metabolic disorders are associated with chronic inflammation that is predominantly due to inflammatory mediators produced by macrophages in adipose tissue, understanding the role of PTP1B in controlling the homeostasis of anti- and pro-inflammatory responses in macrophages is essential to predict the effects of these inhibitors. What was known thus far was that mice with myeloid-specific deletion of PTP1B on a high fat diet are protected from inflammation, hyperinsulinemia and endotoxemia induced by LPS (139). This indicates that there is true potential for a PTP1B

inhibitor to combat the chronic inflammation observed in obese and diabetic patients, particularly if the focus of drug delivery involves macrophages. Nonetheless, we have to keep in mind that although we have shown that PTP1B inhibition enhances the anti-inflammatory response through IL-10/STAT3 signaling, we also observed that it could be converted to a pro-inflammatory response due to upregulation of IL-10/STAT1 target genes if PTP1B inhibition is prolonged or too efficient (**Figure 4.8**).

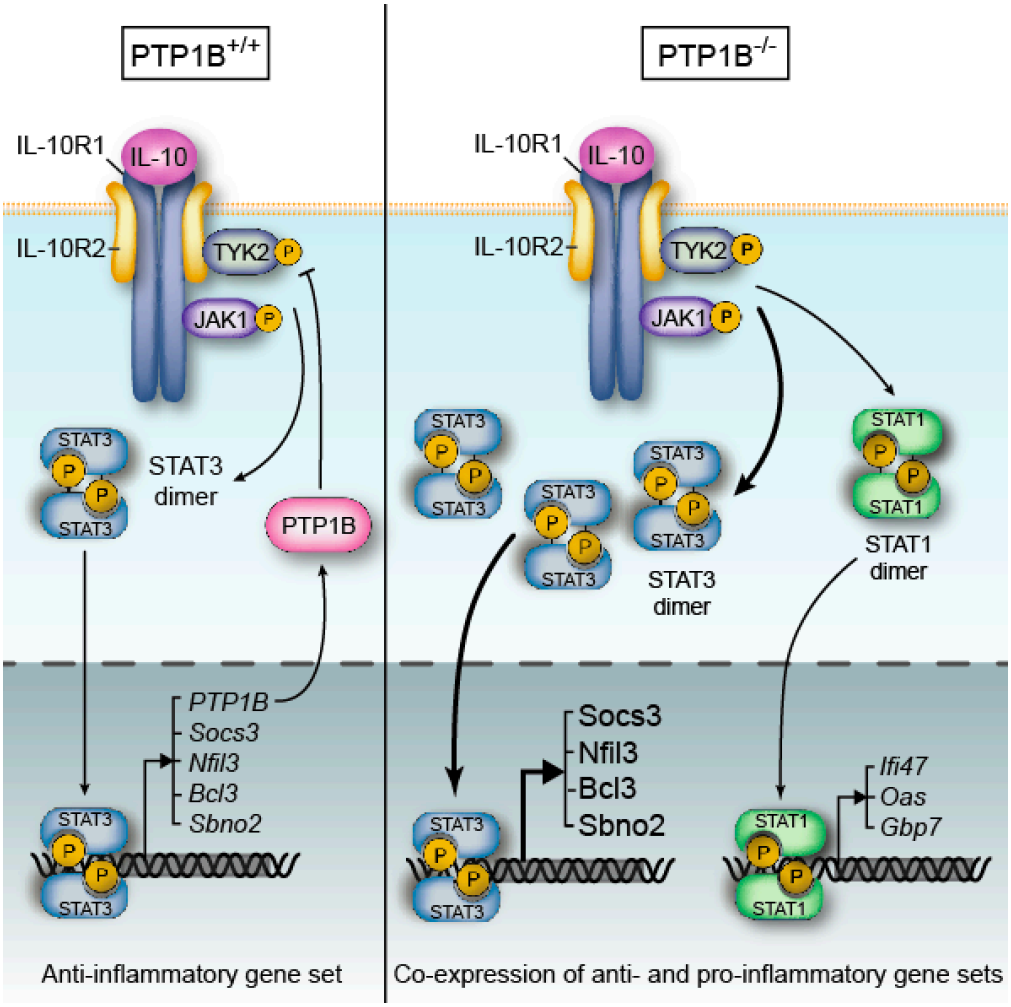

Figure 4.8: PTP1B regulates the IL-10-induced transcriptome in macrophages. Deficiency of PTP1B in macrophages leads to STAT3 hyperphosphorylation in response to IL-10, resulting in increased anti-inflammatory gene expression. STAT1 phosphorylation is also increased in absence of PTP1B and this explains the induction of a pro-inflammatory gene set not observed in PTP1B$^{+/+}$ macrophages upon IL-10 stimulation. Figure from Noriko Uetani.

In this study, we have established that in macrophages, PTP1B sets the threshold against the pro-inflammatory response. We have shown that PTP1B is not required for the inhibition of IL-6R pro-inflammatory downstream signaling, and this may be due to the fact that IL-6R uses more JAK1 than JAK2 and is thus not inhibited by PTP1B. Furthermore, unlike the IL-6R, IL-10R does not contain a SOCS3-binding site so it is not susceptible to inhibition by SOCS3 that is induced with this anti-inflammatory response (274). It is important to note that a prolonged anti-inflammatory response is detrimental to the host, so in future studies or potential drug treatments it will be critical to ensure the proper dosage of PTP1B so that its activity is maintained below a certain threshold and that there is a balance between anti- and pro-inflammatory responses.

CHAPTER 5

GENERAL DISCUSSION

There is still much to learn regarding the mechanisms that govern cancer initiation and progression, as these vary amongst different types of cancer. It is crucial to attempt to elucidate these mechanisms if we hope to one day be able to treat or even cure cancer. What we have discovered over the decades of cancer research is that there are certain acquired cellular characteristics that are so common across the various cancer types that they have been become “hallmarks” of cancer. Two of the newest hallmarks of cancer that are the topic of this thesis are dysregulated cellular bioenergetics and tumour-promoting inflammation. In the Tremblay lab, we study protein tyrosine phosphatases (PTPs) and their role in modulating cancer progression. We and others have discovered that several PTPs regulate different types of cancer. Some act as oncogenes, some act as tumour-suppressors, and others can play both roles, depending on the cancer type (10, 308). Due to their involvement in a plethora of cancers, this thesis investigated the role of PTPs in regulating cancer cell metabolism and tumour-promoting inflammation.

In Chapter 2, I investigated the role of PTPs in regulating cancer bioenergetics of colorectal cancer cells. I established that 24 PTPs out of the hundreds of members in the superfamily modulate colorectal cancer cell metabolism: 16 were positive regulators of mitochondrial respiration and 8 were negative regulators. I chose one of these candidates, TC-PTP, and further validated its role in promoting mitochondrial respiration and ATP production, as well as in repressing STAT1, STAT3 and p38 activation.

In Chapter 3, I sought to determine the role of TC-PTP in promoting the polarization of macrophages and discovered that it is a negative regulator of M1 pro-inflammatory macrophage polarization. TC-PTP modulates the transcriptome of M1 macrophages and downregulates the expression of genes involved in the immune response, macrophage

functions and metabolic pathways. Furthermore, I showed that TC-PTP-deficient macrophages derived from a myeloid-specific mouse model have a dysregulated cytokine production. By comparing the systemic and myeloid-specific mouse models of TC-PTP deletion, I also observed that TC-PTP can have both a cell-intrinsic and cell-extrinsic role in promoting macrophage polarization and immune response.

In Chapter 4, we demonstrated that PTP1B is a negative regulator of the IL-10-induced transcriptional program in macrophages. Deficiency of PTP1B in macrophages leads to transcriptional changes observed predominantly as an induction of anti-inflammatory genes, with an aberrant upregulation of a STAT1-induced pro-inflammatory gene subset. PTP1B sets the threshold to maintain a homeostasis between pro- and anti-inflammatory immune responses.

5.1 IFN-ɣ-STAT1-STAT3-p38 signaling pathway

We found intriguing that in both colorectal cancer cells and bone marrow-derived macrophages, loss of TC-PTP led to hyperactivation of STAT3 and p38. While we have yet to study it in macrophages, we also observed an increase in STAT1 phosphorylation in colorectal cancer cells. Considering that p38 is known to be involved in cell survival, that STAT3 is often overexpressed in cancer and that STAT1 is induced upon IFN-ɣ stimulation, we wondered whether these proteins could somehow be related in both our models that could explain the dual link of TC-PTP in cellular metabolism and macrophage immune responses. An exciting study by Zha and colleagues established that IFN-ɣ is a master regulator of cytokine signaling. They proposed that this occurs in a two-step process: 1) IFN-ɣ downregulates the cytokine-induced activation of STAT3 and upregulates STAT1 signaling, and 2) IFN-ɣ induces the internalization of gp130, a subunit

common to many cytokine receptors, through the activation of p38 (309). Regarding the first strategy by which IFN-ɣ acts as a master regulator, it is known that it also inhibits IL-6 functions and signaling, thereby further ensuring the inhibition of cytokine-induced STAT3 signaling. Interestingly, different STAT proteins can antagonize each other by blocking the formation of active complexes for transcription (310). Given that STAT proteins can form heterodimers, a protein that is upregulated could form a heterodimer with other STAT proteins to inhibit its proper activation. For instance, increased phosphorylated STAT1 proteins could form dimers with STAT3, thus making the phosphorylated STAT3 less available to form homodimers with other STAT3 proteins and ultimately decreasing their translocation to the nucleus. It is also possible that STAT1 competes with STAT3 for docking sites on the IFNGR since they are recruited by the same SH2 domains, again dampening STAT3 activity (309).

This study also demonstrated that acute stimulation with IFN-ɣ results in hyperphosphorylation of p38 and gp130 (309). Active p38 phosphorylates gp130 on Ser782, which allows for its internalization (311, 312). One of the main cytokine receptors that utilizes gp130 is the IL-6R (140, 274), so the internalization of this subunit will inhibit IL-6R and consequently downstream STAT3 signaling. It is unclear whether p38 activation is also dependent on the induction of STAT1, but it has been shown that they at the very least act in parallel to inhibit STAT3 transcriptional activity, emphasizing the function of IFN-ɣ as a master switch of cytokine signaling.

In our CRC and macrophage models, there also seems to be a cooperation between IFN-ɣ, STAT1, STAT3 and p38. In TC-PTP-deficient CRC cells, both STAT1 and STAT3 are hyperphosphorylated, as well as p38. However, we have not analyzed the STAT1

and STA3 downstream gene targets in these cells, so we cannot say if one pathway is preferentially activated over the other. Although IFN-ɣ has been shown to negatively regulate STAT3 expression through p38 (309), another study has demonstrated that p38 can actually promote STAT3 transcriptional activity upon stimulation with IL-6 (190). Hence, p38 could either activate or inhibit the transcriptional activity of STAT3, depending on the microenvironmental cues. As such, it would be interesting to stimulate TC-PTP colorectal cancer cells with IFN-ɣ or IL-6 and observe the effect on STAT3 phosphorylation and activity. Doing so should enable us to discover whether there is a similar IFNGR-STAT1-p38 complex that is favoured over STAT3 cytokine signaling in TC-PTP-deficient cancer cells. In TC-PTP-deficient macrophages stimulated with IFN-ɣ, we observed an increase in STAT3 and p38 phosphorylation. The next important step in this study will be to analyze the expression and activity of STAT1 in these cells and determine whether the STAT1 transcriptional program is favoured in these macrophages to induce the expression of pro-inflammatory genes. It is possible that an IFNGR-STAT1-p38 regulatory complex exists in both our colorectal cancer cells and bone marrow-derived macrophages, placing TC-PTP as a central player in the regulation of this pathway.

5.2 STAT3 and HIF-1α as regulators of aerobic glycolysis

Inhibition of TC-PTP in colorectal cancer cells results in hyperphosphorylation of its substrate STAT3 and decreased mitochondrial respiration, but we do not yet know the mechanism by which loss of TC-PTP leads to altered cellular metabolism. As mentioned previously, several cancers overexpress STAT3. A study has shown that STAT3 exerts its pro-oncogenic properties in part by inducing aerobic glycolysis, since inhibition of

phosphorylated STAT3 in various tumour cell lines results in a decrease in glycolysis, followed by cancer cell growth arrest and cell death (188). The authors further demonstrated that constitutive activation of STAT3 increased glycolysis in a HIF-1α-dependent manner and downregulated mitochondrial respiration. Inhibition of STAT3 in STAT3-dependent cancer cells lines resulted in a decrease in HIF-1α as well as lactate production, further emphasizing the importance of HIF-1α in promoting aerobic glycolysis in presence of STAT3. Considering that constitutive STAT3 seems to be a positive regulator of the Warburg effect in these cells, it could explain in part why so many cancers are addicted to STAT3: they depend on this metabolic switch to thrive and for tumourigenesis (188, 313-316).

As a master regulator of cellular responses to hypoxia, HIF-1α is essential in regulating cellular metabolism to overcome this stress. As we demonstrated in Chapter 2, TC-PTP-deficient colorectal cancer cells were unable to overcome a stress, as was determined by the inability of the cells to perform mitochondrial respiration after FCCP stimulation. It will be important to consider the expression level and activity of HIF-1α in these cells. Is it possible that loss of TC-PTP in these cells induces transcriptional changes, thereby repressing HIF-1α activity and diminishing the metabolic switch to aerobic glycolysis? Similarly, would overexpressing STAT3 in TC-PTP-deficient cells promote aerobic glycolysis and synergize with the loss of TC-PTP to further impair mitochondrial function? These would be essential experiments to perform to better understand the role of TC-PTP in regulating cellular metabolism.

5.3 Macrophages in colorectal cancer

One of the main risk factors for colorectal cancer is inflammation (108, 112, 113). Inflammation of the colon, or colitis, is often the result of bacterial infections or other invading pathogens, which disrupt the intestinal epithelial cell barrier of the colon and allow for the entry of pathogens inside the gut lumen. This bacterial invasion triggers an inflammatory cascade as a result of the activation and recruitment of macrophages, neutrophils and other immune cells to the site of injury to combat the pathogen (93). To understand this particular colorectal cancer risk factor, one must also comprehend the origin of inflammation of the colon that can lead to IBD or to colitis-associated colorectal cancer (CAC). Apart from their major functions in the innate and acquire immune systems as well as killing invading pathogens, macrophages also play a role in regulating iron homeostasis. (317-319).

5.4 Macrophages regulate iron homeostasis

In our bone marrow-derived macrophages derived from TC-PTP knockout mice, we observed a drastic downregulation of *Ftl1*, a gene involved in iron homeostasis. To try to make sense of this, we must first understand how iron and macrophages are interconnected. Although iron is important for the differentiation and proliferation of immune cells such as macrophages, it also affects the antimicrobial immune function of macrophages through pathways induced by IFN-ɣ activation (320-323). Macrophages maintain iron homeostasis at two levels. First, there is a specialized population of tissue-resident macrophages in the bone marrow that are required to support erythropoiesis, phagocytosing and digesting the nuclei of erythroblasts, as well as delivering the iron and heme required to support hemoglobin synthesis. At the second level, macrophages in the

spleen, bone marrow and liver engulf senescent red blood cells which contain the majority of iron required to support heme biosynthesis in erythroblasts (319, 324). Essentially, macrophages are heavily implicated in regulating iron levels and they do so by recovering iron from old senescent red blood cells and returning it to the circulation (323). Considering that iron is beneficial for the proliferation and pathogenicity of invading pathogens, macrophages help to limit pathogen growth by controlling the intracellular iron available and sequestering it (323, 325).

5.4.1 Iron as a regulator of cellular metabolism

Iron is also implicated in the modulation of cellular metabolism. This process is indirect and is a consequence of the immune response to pathogens. In the context of infection, macrophages recognize LPS present at the surface of certain bacteria by TLR4. Upon activation of the IFNG receptor, expression of NOS2 in macrophages is induced through activation of STAT1 and NF-κB pathways (319, 326). Nitric oxide (NO) will be produced following NOS2 upregulation and will interact with the heme-iron in complex IV of the ETC to act as a physiological inhibitor of mitochondrial respiration while also promoting ROS production (327). Inflammation resulting from invading pathogens in the colon can favour aerobic glycolysis by inhibiting mitochondrial respiration as the result of NO production (319). On the other hand, iron accumulation in macrophages inhibits *NOS2* transcription due to inhibition of STAT proteins, particularly STAT1, in response to IFN-ɣ signaling (328, 329). It seems evident that iron is important not only in the macrophage response to invading pathogens that could lead to colitis and eventually perhaps CRC, but also modulates cellular metabolism by inhibiting components of the ETC.

5.4.2 Iron and macrophage polarization

Iron and the polarization of macrophages seem to be intrinsically linked. While the polarization of macrophages can modulate iron metabolism, iron can also directly influence macrophage polarization (323, 330). We know that pro-inflammatory M1 macrophages have an enhanced microbicidal capacity, secrete high levels of pro-inflammatory cytokines, and produce great amounts of oxygen and nitrogen radicals to increase their killing activity (323, 331, 332). The retention of iron in M1 macrophages reduces the amount of circulating iron levels and thus the availability of the essential nutrient for pathogens (323). This appears to be one of the strategies that macrophages use to combat infections. However, there seems to be a required threshold regarding the sequestered iron permitted for proper macrophage functions. Clinical data has demonstrated that iron overload in macrophages, whether as a result of hemolysis or iron supplementation, interferes with their antimicrobial activity (318). In addition, a range of intracellular microbes, including *Salmonella typhimurium*, have been shown to resist killing by macrophages upon phagocytosis by gaining access to their iron pools (333, 334). In addition, when iron is in surplus in macrophages exposed to IFN-ɣ, such as is the case for M1 macrophages, it has been shown to directly inhibit the binding of HIF-1α, thereby affecting cellular metabolism and *NOS2* transcription (322, 335, 336). In this case, when macrophages are overwhelmed by iron, repression of NOS2 transcription by HIF-1α inhibits NO production, worsening antimicrobial activity by macrophages (318, 336, 337). It thus seems essential to for macrophage to tightly regulate iron homeostasis for their proper functioning and response to bacterial infections. Accumulation of some iron in macrophages seems to be beneficial since it leads to production of NO and favours bacterial killing. It also makes free iron less available for pathogens to thrive and become

more pathogenic. However, too much iron sequestered in macrophages seems to be detrimental to their capacity to combat infections. Excess iron will inhibit HIF-1α downstream of the IFNG receptor, leading to inhibition of NO production, diminishing bacterial killing and decreasing mitochondrial respiration. These various reports highlight a role for HIF-1α as a central regulator of macrophage iron homeostasis, immunity and cellular metabolism due to its sensitivity to both oxygen and iron.

As mentioned in Chapter 3, the most downregulated gene in TC-PTP knockout M1 macrophages with about a 27-fold decrease was *Ftl1*, a light chain of ferritin. Ferritin allows for the intracellular storage of iron and thus permits macrophages to sequester it (338, 339). A study has demonstrated that iron coincides with inhibition of STAT1 and results in decreased M1 macrophage polarization (323). The strong downregulation of *Ftl1* in TC-PTP knockout M1 macrophages now makes perfect sense in the context of maintaining their polarization status. A we know, macrophages are very dynamic and can change from one inflammatory state to another. Lower expression of ferritin in KO macrophages means that *NOS2* expression will not be repressed and they will be able to polarize towards M1 macrophages more effectively, so it is possible that TC-PTP-deficient macrophages reprogram their transcriptome to repress genes involved in iron storage and import, thus promoting *NOS2* expression, M1 macrophage polarization and possibly increased resistance to bacterial infections since the phagocytosed pathogens will not be able to utilize iron pools within the macrophages as effectively. Now whether all these events are the result of TC-PTP deficiency remains to be seen. However, it could very well be that TC-PTP deficiency in macrophages upregulates STAT1, which would induce pro-inflammatory gene expression and inhibit transcription of genes involved in

iron storage and metabolism. Hence, in our Th1 *in vivo* infection mouse model with TC-PTP mice, it is likely that the knockout mice will be more resistant to *Salmonella typhimurium* infection due to their low iron storage and high secretion of pro-inflammatory cytokines. We will have to elucidate what the effect of loss of TC-PTP will be in on cellular metabolism. The previous studies would suggest that lower iron in TC-PTP-deficient macrophages would have a decreased mitochondrial respiration since high levels of NO will be produced, which can then inhibit complex IV of the ETC. In summary, various biological processes in macrophages, including immune responses, antimicrobial activity, cellular respiration and iron metabolism, seem to be interconnected and regulated. Whether TC-PTP is the master regulator of these pathways remains to be seen, but RNA-Seq, qRT-PCR and *in vivo* infection models should help us further understand the complex pathways and mechanisms regulating these biological processes.

In Chapter 4, we established that PTP1B, which is closely linked to TC-PTP, is a negative regulator of the macrophage transcriptional program and immune responses upon induction with IL-10. PTP1B is essential to maintain a balance between pro- and anti-inflammatory cytokines since its deficiency promotes a predominantly anti-inflammatory response induced by STAT3 activation and upregulates a subset of STAT1-induced pro-inflammatory genes. Again, we observed this interplay between STAT1 and STAT3 signaling pathways. Considering that TC-PTP and PTP1B are closely related, have similar substrate specificities and are both involved in macrophage development and functions (206, 340), it would be very interesting to study them in parallel in the context of macrophage polarization, resistance to bacterial infection, metabolic activity and in regulation of colitis-associated colorectal cancer. PTP1B

knockout mice are not overwhelmed by IFN-ɣ as TC-PTP knockout mice are, so it would be interesting to establish whether PTP1B macrophages will be more apt at polarizing towards M1 macrophage, and if this would affect their cellular metabolism and mitochondrial functions.

CONCLUSION

As the time passes, we understand more and more about the different processes that regulate the initiation and progression of cancer. Inflammation is crucial for cancer cells to thrive as well as for the regulation of cellular bioenergetics. These two pathways regulate each other, and as has been discussed, also regulate iron storage and metabolism in macrophages. In this thesis, I have answered my objectives. I have shown that several members of the PTP superfamily regulate cellular metabolism of colorectal cancer cells and that TC-PTP positively regulates mitochondrial respiration and ATP production. I have also demonstrated that TC-PTP negatively regulates pro-inflammatory macrophage polarization and that myeloid-specific deletion of TC-PTP is sufficient to regulate this process as well. Finally, we have showed that the closely-related PTP1B negatively regulates IL-10-induced transcriptional reprogramming and anti-inflammatory responses in macrophages.

The data obtained and demonstrated in this thesis are of important value not only to understand how PTPs regulate metabolism and immune responses and how these are interlinked, but also as ideas for potential treatment for IBD, CAC or CRC. If we can confirm that TC-PTP-deficient macrophages do not promote the Warburg effect, macrophage immunotherapies could be a viable treatment option. In cancer, we want to favour M1 macrophage polarization since these have anti-tumoural functions and can favour tumour regression. Given that TC-PTP deficiency promotes M1 macrophage polarization both in a cell-intrinsic and cell-extrinsic manner, we could inhibit it directly in macrophages derived from the patient, promote their polarization towards pro-inflammatory macrophages are re-inject them into the patients. By doing so, in theory,

these M1 macrophages should be able to help fight cancer cells by reducing the number of TAMs in the tumour microenvironment and enabling their switch to M1 macrophages.

Albeit I have answered many questions in my doctoral thesis, I have uncovered even more questions that demand to be answered. *Does TC-PTP inhibit mitochondrial respiration of CRC cells through a STAT1-HIF-1α-dependent mechanism? Does TC-PTP regulate the metabolism of macrophages? If so, it is a cause or a consequence of altered immune response and iron homeostasis? Does PTP1B alter macrophage metabolism?* Such is the mission of a scientist: always be skeptical and answer questions with more questions. I have learned and acquired a tremendous amount of knowledge through my PhD, but there is still much to learn. The quest for knowledge never ceases.

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