Investigating The Role of Prohibitin in The Metabolism and Immune Function of Macrophages

by

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ABSTRACT

Prohibitin (PHB) is an evolutionarily conserved protein capable of modulating the metabolic status of multiple cell types. Phosphorylation of PHB at tyrosine residues regulates signal transduction and intracellular trafficking by hormones, growth factors and antigen-activated immune cells. Our laboratory has developed two transgenic mouse models by overexpressing PHB and a mutated PHB (Y114F-PHB) under the fatty acid-binding protein 4 (*Fabp4*) gene promoter, which is selectively expressed in adipocytes and monocytic macrophages. Based on discoveries from these two transgenic mouse models named Mito-Ob and mutant-Mito-Ob, I speculate that PHB plays an important role in modulating macrophage polarization and function in part through its Tyr-114 phosphorylation (**Chapter 2**). Macrophages can polarize into two major phenotypes: proinflammatory (M1) and anti-inflammatory (M2). This functional plasticity of macrophages is heavily influenced by internal and external signals, which alter their survival and output. In this thesis, I examined the role of PHB in the metabolism and immune function of macrophages. The importance of PHB tyrosine phosphorylation in cell signalling is reviewed in **Chapter 3.** PHB has proven to be a crucial link in endocrine-immune crosstalk. **In Chapter 4**, the immunometabolic aspect of PHB function is reviewed in the context of Mito-Ob and mutant-Mito-Ob mice based on their metabolic and immune dysregulations. While PHB function was examined in adipocytes during my master's thesis, its role in macrophages remains unexplored. In **Chapter 5,** I investigated the role of PHB under the M1/M2 dichotomy in a murine cell line, RAW 264.7, and the Mito-Ob and mutant-Mito-Ob mouse models. PHB's involvement in the functional plasticity of macrophages is many folds. Overexpression of PHB in macrophages differentially affected cytokine production in M1 and M2 macrophages *in vitro*. Mutation of the Tyr-114 phosphorylation site in PHB affected ERK and STAT6 signalling, arginase synthesis and activity, and

mitochondrial respiration. In summary, PHB plays a crucial role in integrating cell signalling events with metabolic switches, as uncovered in polarized macrophages. The results of this thesis support the notion that PHB has important functions in regulating immune cell types, and further investigation on targeting PHB or PHB-related pathways in immune cells may yield therapeutic potential.

ACKNOWLEDGEMENTS

Life has a way of surprising you when you least expect it. In the years I've been a Ph.D. candidate, I have transitioned from my late 20s to my early 30s. As an academic community, we celebrated awards, promotions and graduations but also endure losses. Through it all, I learned the lesson of perseverance. I am graduating with more than a degree but a drive to continue learning and advancing my understanding of the world as we know it.

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LIST OF ABBREVIATIONS

(k)Da	(kilo)Dalton
(m)RNA	Messenger ribonucleic acid
aa	Amino acid
Akt/PKB	Ak strain transforming/ Protein kinase B
aP2/Fabp4	Adipocyte protein 2/ Fatty acid binding protein 4
ATP	Adenosine triphosphate
BAP	B-cell receptor-associated protein
Brg1/Brm	Brahma-related gene 1/ Brahma
BSA	Bovine serum albumin
CCL	C-C motif ligand
CD	Cluster of differentiation
CoA	Coenzyme A
CSF1R	Colony-stimulating factor-1
CXCL	C-X-C motif ligand
Cys or C	Cysteine
DNA	Deoxyribonucleic acid
E2	Estradiol
ECAR	Extracellular acidification rate
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FAO	Fatty acid oxidation
FCCP	Trifluoromethoxy carbonylcyanide phenylhydrazone
GlcNAc	N-acetylglucosamine
GM-CSF	Granulocyte macrophage colony stimulating factor
Grb2	Receptor-bound protein 2

h	Hour(s)
HDAC	Histone deacetylase
HP1	Heterochromatin protein 1
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRS	Insulin receptor substrate
LPS	Lipopolysaccharide
M0	Naïve macrophage
M1	Classically activated/proinflammatory macrophage
M2	Alternatively activated/anti-inflammatory macrophage
MAPK	Mitogen-activated protein kinase
MEK	MAPK kinase/Extracellular signal-regulated kinase
MCP1	Monocyte chemoattractant protein 1
MHC	Major histocompatibility complex
MIG7	Migration inducting gene 7
MPS	Mononuclear phagocytic system
NADP(H)	Nicotinamide adenine dinucleotide phosphate (with hydrogen; reduced)
NFκB	Nuclear factor kappa B
NK	Natural killer
NO	Nitric oxide
Nrf2	Nuclear factor erythroid-2-related factor 2
O-GlcNAc	*O*-linked β-N-acetylglucosamine
OCR	Oxygen consumption rate
OGT	*O*-GlcNAc transferase

OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffered saline
PGC	PPAR gamma coactivator
PHB/PHB1	Prohibitin/prohibitin 1
PHB2/REA	Prohibitin 2/Repressor of estrogen receptor activity
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PMA	Phorbol 12-myristate 13-acetate
PPAR	Peroxisome proliferator-activated receptor
PPP	Pentose phosphate pathway
PX	Phox
Rb	Retinoblastoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction
SEM	Scanning electron microscope
Ser or S	Serine
Shc	Src homology 2 domain containing
Shp1	Protein tyrosine phosphatase 1
SPFH	Stomatin-prohibitin-flotillin-hflc/K
STAT	Signal transducer and activator of transcription
T (cell)	Thymus
TAM	Tumour-associated macrophage
TCA	Tricarboxylic acid
TGFβ	Transforming growth factor beta

Th	Thymus helper
Thr or T	Threonine
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
Tyr or Y	Tyrosine
UDP	Uridine diphosphate
µM	Micromolar
UTR	Untranslated region
Wnt	Wingless/integrated

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Prohibitin: a potential therapeutic target in tyrosine kinase signaling.

Sudharsana Rao Ande, Yang Xin Zi Xu & Suresh Mishra

Signal Transduction and Targeted Therapy volume 2:17059. doi: 10.1038/sigtrans.2017.59.

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Prohibitin: A new player in immunometabolism and in linking obesity and inflammation with cancer.

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Prohibitin plays a role in the functional plasticity of macrophages.

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CHAPTER 1. INTRODUCTION

1.1 Prohibitin (PHB)

1.1.1 The *PHB* gene and mRNA

The *PHB* gene is evolutionarily conserved from yeasts to humans and ubiquitously expressed [1]. It was discovered during a microinjection assay, where researchers isolated and fractionated rat liver mRNAs based on density gradient and tested each fraction on serum-restricted human diploid fibroblasts in search of inhibitors of proliferative activity [2,3]. One such candidate was found to inhibit cell cycle progression and was given the name "prohibitin" [4]. In humans, the *PHB* gene (PubMed Gene ID: 5245) is located at 17q21.33. This region of the genome is linked to susceptibilities to early-onset breast cancer (17q21-q22), autoimmune diseases such as type 1 diabetes (17q21.2, where the STAT3 gene is located) and the propensity to accumulate abdominal fat [5,6]. Near the *PHB* gene, locus 17q21.1 is associated with genes involved in the regulation of sex steroid levels. Polymorphism in the coding and 3' untranslated regions (3'UTR) of *PHB* is associated with a risk of gastric, ovarian or breast cancers [7–10]. Specifically, the single-nucleotide polymorphism of C to T at nucleotide 729 in the 3'UTR of *PHB* was detected in both gastric cancer and breast cancer patients, but it only appears to increase the risk factor for breast cancer [8,9]. Global knockout of *Phb* in animal models led to lethal phenotypes, indicating that they have fundamentally important functions [11,12]. Liver-specific deletion of *Phb* in mice caused swelling of mitochondria in hepatocytes and the upregulation of genes associated with proliferation, malignant transformation, and liver fibrosis, with some mice developing hepatocellular carcinoma [13].

The *PHB* transcript contains up to 8 exons in humans and 7 exons in mice (*PHB2* mRNA contains up to 10 exons in both humans and mice) [14,15]. Comparative studies found that both the regulatory regions (minus 350 nucleotides), exon lengths and the exon-intron boundaries in rat and human *PHB* are highly conserved, but the intron sizes are generally longer in rats compared to humans [16,17]. The human *PHB* gene is alternatively spliced. In an early study conducted in 1996, researchers identified two *PHB* transcripts: A 1.9-kilobase (kb) transcript and a 1.2-kb transcript [18]. These two transcripts code for the same 30 kilo-Dalton (kDa) protein but differed by their 3'UTR lengths due to alternative polyadenylation [19]. Intriguingly, the research group found several single base mutations unique to the 1.9-kb transcript, including position 729 (C to T) in T98G cells, that led to a loss of *PHB*'s tumour suppressor activity [18]. These observations provided important evidence that the anti-proliferative function of *PHB* is in part executed at the RNA level, likely as a trans-acting regulatory RNA. Others have identified different-sized transcripts of *PHB* at a specific developmental stage or in different experimental systems [20,21]. Subsequently, *PHB* mRNA and protein were both shown to modulate G1 to S transition in cell division and cellular senescence through transcriptional and posttranslational mechanisms [22].

1.1.2 The *PHB2* gene, mRNA and protein

PHB2 is a protein homologue of PHB that shares many of PHB's properties, including ubiquitous expression, multiple subcellular locations, and evolutionary sequence conservation [23]. The human *PHB2* gene is located at 12p13.31 [24]. Polymorphism in exon 9 in *PHB2* leads to reduced reactive oxygen species (ROS) tolerance in the kidneys, which suggests increased susceptibility to nephrotic syndrome [25]. Like the global knockout of *Phb*, the global knockout of *Phb2* in animal models is embryonically lethal [11]. Interestingly, depletion of *Phb2* in cells is accompanied by the loss of PHB at the protein level, while transcription of *Phb* proceeds

irrespective of the presence of *Phb2* [26–28]. Again, similar to liver-specific deletion of *Phb* in mice, hepatocyte-specific *Phb2* knockout in mice caused hepatocyte-specific mitochondrial fragmentation and increased apoptosis, which progressed to extensive liver damage, dramatic imbalance of lipid storage and death [28]. Other tissue-specific knockouts of *Phb2* also produced severe phenotypes and were frequently accompanied by a reduced level of the other gene transcripts [27,29–31]. The PHB2 protein is also known to be a transcriptional co-regulator of estrogen receptors in the nucleus and thus given another name, REA (Repressor of Estrogen Receptor Activity) [32].

1.1.3 The PHB protein

The PHB protein sequence contains 272 amino acids and has a molecular weight of 30 kDa [Figure 1.1]. The protein half-life was reported to be approximately 22 hours *in vitro* in human diploid fibroblasts [16]. Mice and rats share an identical PHB protein sequence, and they differ from the human PHB by one amino acid at position 107 (Phenylalanine in humans and tyrosine in mice/rats) [16]. The total expression of PHB protein varies from tissue to tissue but generally parallels the level of mRNA expression. PHB is a member of the stomatin/PHB/flotillin/HflK/C (SPFH) superfamily, which harbours the conserved PHB domain (also known as the SPFH or Band 7 domain) [23] [Figure 1.1]. Although little is known about the function of this domain, proteins in this superfamily tend to assemble into membrane-bound oligomers that form putative scaffolds and are involved in the formation of lipid rafts [33]. The PHB protein has a hydrophobic region at the N-terminus. Unlike PHB2, which contains an uncleavable mitochondrial targeting sequence at the N-terminus [34], PHB does not possess a typical mitochondrial localization signal in its sequence. Given that PHB forms part of a well-established multimeric complex in the mitochondria, its translocation to the mitochondria is necessary and could be achieved through an unidentified

targeting signal or post-translational modifications. The C-terminus of PHB contains a coiled-coil domain that facilitates protein-protein interactions, with partners such as PHB2 [23]. It also contains a leucine-rich nuclear export region for possible translocation into and out of the nucleus [23].

In yeasts and mammalian cells, evidence show PHB/PHB2 interdependence for protein stability by heterodimerization [26,35]. Knockdown of either protein reduces the other one significantly in a wide range of cell types [26–29,36]. The PHB and PHB2 proteins share about 53% homology, including many crucial post-translational modification sites [Figure 1.1].

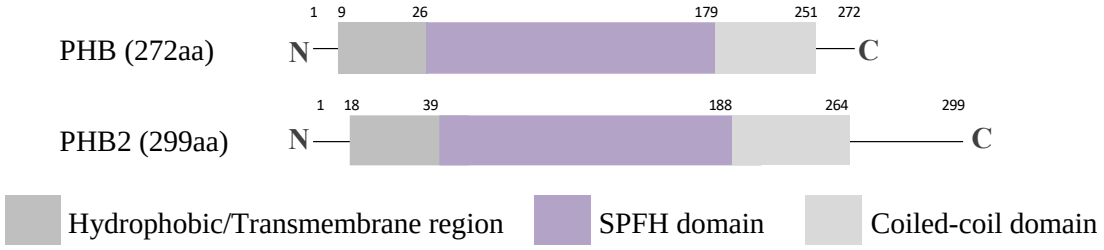


Figure 1.1. The human prohibitin (PHB) and prohibitin 2 (PHB2) protein sequences. Domain structures of PHB and PHB2. Both proteins have similar domains, including a hydrophobic region (dark gray), the conserved stomatin/PHB/flotillin/HflK/C (SPFH or PHB) domain (purple) and a coiled-coil domain (light gray). Boundaries are labelled with amino acid (aa) residue numbers. PHB contains 272 aa and PHB2 contains 299 aa.

PHB has been reported in different subcellular locations, including the mitochondria, plasma membrane, and nucleus, as well as in secreted forms. Trafficking of PHB between different subcellular compartments has also been observed. In the following sections, functions of PHB (and PHB2, to a lesser extent) in each location and known translocation from and to other organelles, will be discussed.

1.2 PHB in the Mitochondria

The function of PHB in the mitochondria has been the best studied and most extensively reviewed [37–40]. In the mitochondria, PHB and PHB2 form a multimeric, macromolecular ring structure within the inner mitochondrial membrane [Figure 1.2]. Their interdependence is the most apparent in this organelle. The PHB complex, made up of 12-16 alternating PHB/PHB2 heterodimers of more than 1 MDa in molecular weight, has been identified in yeasts [41], *Caenorhabditis elegans* [42] and mammals [35]. The reported functions of this complex include protein scaffolding, lipid metabolism, and mitochondrial metabolism, including bioenergetics, biogenesis, and stability. Specifically, the PHB complex interacts and inhibits mitochondrial-AAA [43] and OMA1 proteases [44], which in turn promotes mitochondrial protein stability and regulates the cellular stress response. Examples of stabilized proteins include mitochondrial transcription factor A (TFAM) [45] and optic atrophy 1 (OPA1) [26]. Loss of either PHB or PHB2 increases the generation of reactive oxygen species (ROS), abnormal mitochondrial cristae and an increased sensitivity towards stimuli-elicited apoptosis [46]. Fascinating results have been discovered in C. *elegans*, where the knockdown of the PHB complex shortened the lifespan of the wild-type nematodes but dramatically increased the survival of nematodes under dietary restriction or with a compromised metabolism, such as the diapause mutant, daf-2 [47]. It was later shown that PHB depletion led to strong upregulation of mitochondrial unfolded protein response in the wild-type, but the response was reduced in the daf-2 mutant. The difference in mitochondrial unfolded protein response was responsible for the difference in longevity observed between the two phenotypes [48]. In a separate study, stimulation of the mitochondrial unfolded protein response in skeletal muscle stem cells was coupled with PHB protein synthesis; the result led to a reduced cell senescence [49]. In cell types that heavily depend on the mitochondria during cell differentiation, increased levels of both PHB

and PHB2 have been observed, such as in adipocytes [50]. On the other hand, the knockdown of either protein results in reduced adipogenic markers and a reduction of mitochondrial biogenesis [50].

1.3 PHB in the Nucleus

Unlike PHB and PHB2 in the mitochondria, nuclear PHBs seem to function with other nuclear molecules rather than each other. One major function of PHB is transcriptional co-regulation, where it prevents cell proliferation and acts as a tumour suppressor. PHB is found to co-localize with histone deacetylases (HDACs) [51–53], cell cycle-associated proteins [54–57], mitochondrial-nuclear mediator, nuclear factor erythroid 2-related factor 2 (Nrf2) and RNA-binding proteins [58] [Figure 1.2]. PHB and its C-terminal coiled-coil region display growth-suppressive properties through direct repressive interaction with E2F1 and recruitment of HDAC1, p130, Rb, HP1 and Brg1/Brm [51,55–57,59]. HDAC4 is also downstream of PHB regulation, as observed in PHB silencing models having increased nuclear HDAC4-dependent epigenetic changes during cholestatic liver injury [52]. PHB is found to physically interact with p53 in breast cancer cell lines and enhance its transcription activity [54]; this enhancement, however, may be attenuated by Skp2B, an F-box protein that targets PHB for ubiquitin-mediated degradation [60]. Nrf2 is a transcription factor that regulates several nuclear-encoded mitochondrial proteins. PHB is found as an activator of Nrf2 expression in intestinal epithelial cells, which helps prevent inflammatory injury and oxidative stress upon overexpression [58]. Nuclear PHB is also responsible for inhibiting Wnt/β-catenin signalling and decreasing intestinal tumorigenesis in cell lines and mouse models of colorectal cancers [61]. Both PHBs have been independently found to regulate sex-steroid sensitive genes, including repression of androgen receptor-mediated transcription [53].

Both PHBs can shuttle between the nucleus and mitochondria. PHB2, not PHB, was shown to have a nuclear localization sequence at the C terminus (within amino acids 51-299), which responds to the presence of ERα and E2 binding [34]. Androgen and TGFβ treatments also promote PHB export from the nucleus in human prostate cancer cells, which precedes apoptotic cell death [62]. In response to oxidative stress, PHB translocates from mitochondria to the nucleus and behaves as an anti-apoptotic transcriptional regulator [63]. On the other hand, in transformed cell lines, PHB is predominantly located in the nucleus and translocates to the mitochondria in response to apoptotic inducers. In bladder cancer cells, Akt phosphorylation at Thr-258 of PHB precedes PHB translocation from the nucleus to the mitochondria and is also required for cancer cell proliferation [64]. In transformed cell lines, deletion of amino acids 243-272 in PHB prevented its nuclear export in response to apoptotic signal; as a result, a nuclear export signal in PHB is proposed within the region, which contains a leucine/isoleucine-rich motif [65].

1.4 PHB on the Plasma Membrane

PHB on the plasma membrane supports cell-to-cell communication, cell signalling, and acts as an entry target by a wide range of microorganisms, indicating a role of PHB in the molecular events leading up to host infection [Figure 1.2]. For this reason, PHB has been a target of pharmacological intervention, and the various PHB ligands are recently reviewed for their mechanistic action and therapeutic potential [66]. Membrane PHB and PHB2 can either form a complex or act independently during signal transduction. PHB is found to localize with CD3 in activated leukemia T cells, [67,68] FceRI, Lyn and Syk in mast cells [69], and immunoglobulin M (IgM) receptor on B cells [70]. Direct interaction of PHB with RAF1 in epithelial cells is required for the membrane localization, phosphorylation, and activation of RAF1 in the Ras/ RAF1 signalling

pathway and for subsequent cell adhesion and migration [71]. PHB also acts as a signalling molecule; this ability requires various post-translational modifications, including Tyr-114 phosphorylation by insulin [72], Thr-258 phosphorylation by Akt [73], *O*-GlcNAc conjugation at Ser-121 and Thr-258 [74], or palmitoylation at Cys-69 [69,75]. A detailed review of PHB in tyrosine kinase signalling pathways will be discussed in Chapter 3. Cys-69 in PHB is highly conserved in mammals; it is the only cysteine residue in PHB. In mast cells, palmitoylation of Cys-69 facilitates translocation of PHB to the plasma membrane and its interaction with Eps 15 homology domain protein 2 (EHD2), a lipid raft protein [75]. Proteins that are modified by palmitate have enhanced hydrophobicity, and congregate in lipid rafts, which facilitates more efficient signal transduction. Lipid rafts are also important sites during the subcellular trafficking of proteins between membrane compartments. This property also facilitates the retention of proteins within the membrane system without a membrane-targeting sequence.

1.5 Secreted PHB

The secreted form of PHB is the least well-known. Its release into the general circulation suggests potential interaction with the immune system and employment as a biomarker. Using differential immunization, PHB and PHB2 were found at higher levels in the serum of colorectal cancer patients [76]. Similarly, PHB was identified as a tumour antigen released from breast cancer and gastric cancer patients [77,78]. Once released, circulating PHB can bind strongly to component C3 to enhance complement activation and induce lysis of sensitized sheep erythrocytes [Figure 1.2] [79]. In cardiomyocytes challenged with endotoxin and mice with sepsis, PHB acts as a paracrine factor from hepatocytes and exerts antioxidative and anti-inflammatory functions on cardiomyocytes [80]. Recently, PHB was also detected in the extracellular vesicles (known as micro-

vesicles) secreted from neurons and astrocytes [81], though the exact biological process remains elusive.

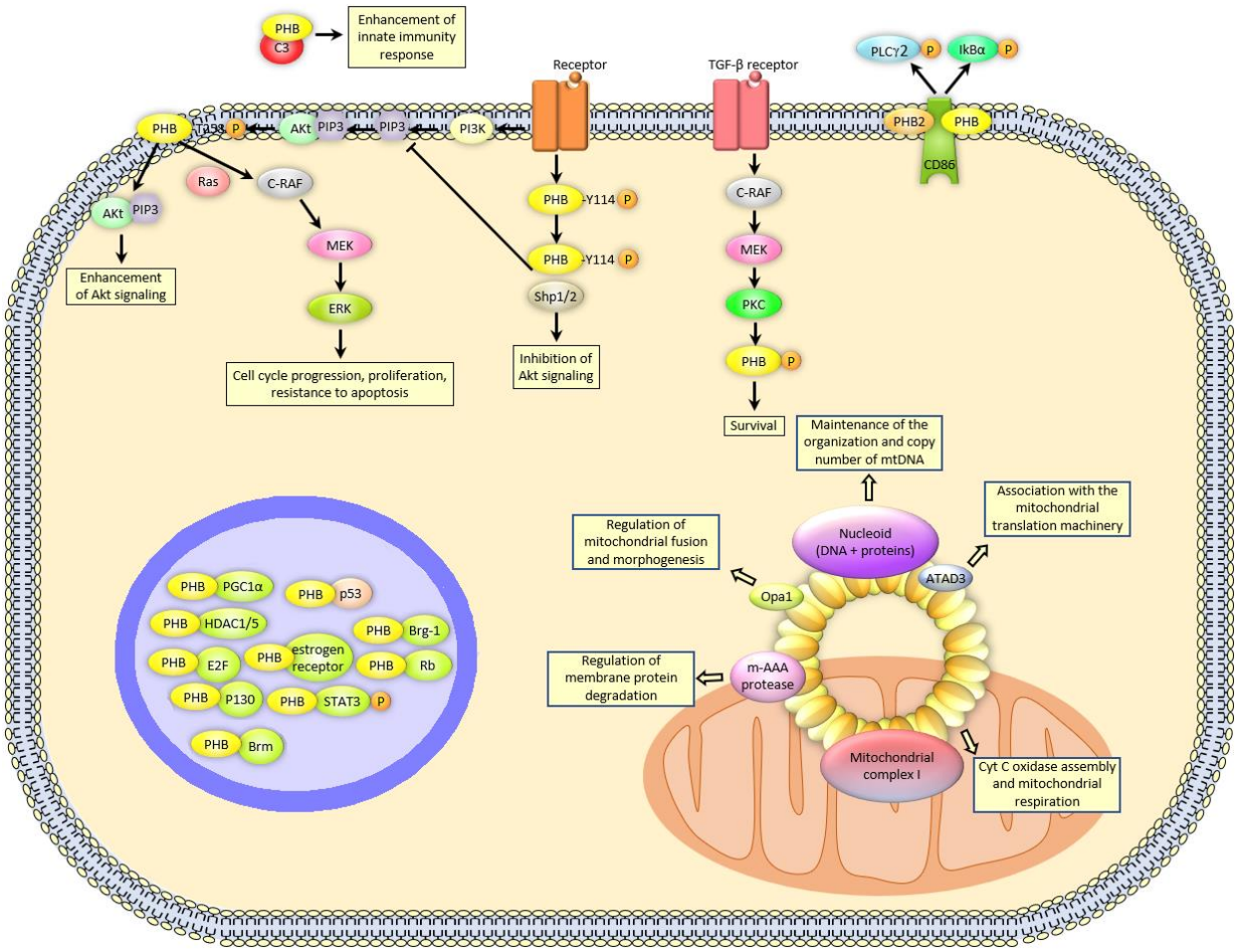


Figure 1.2. Representative functions of PHB in different subcellular compartments, including the mitochondria, nucleus, and plasma membrane of a cell, as well as a secreted form. Image revised based on previously published literature [82]. In the mitochondria, PHB forms a multimeric complex in the inner mitochondrial membrane with PHB2. This complex is known to interact with mitochondrial-AAA, OMA1 proteases, ATAD3 and mitochondrial complex I with an overall goal to maintain mitochondrial stability. In the nucleus, PHB interacts with transcription factors included above to regulate cell proliferation and viability. On the plasma membrane, PHB supports cell-to-cell communication and cell signalling through post-translational modification, such as the phosphorylation of tyrosine (Y)114. The secreted form of PHB may be actively secreted as a signalling molecule in cancer patients or binds strongly to component C3 to enhance complement activation. The last aspect of PHB function is still unclear.

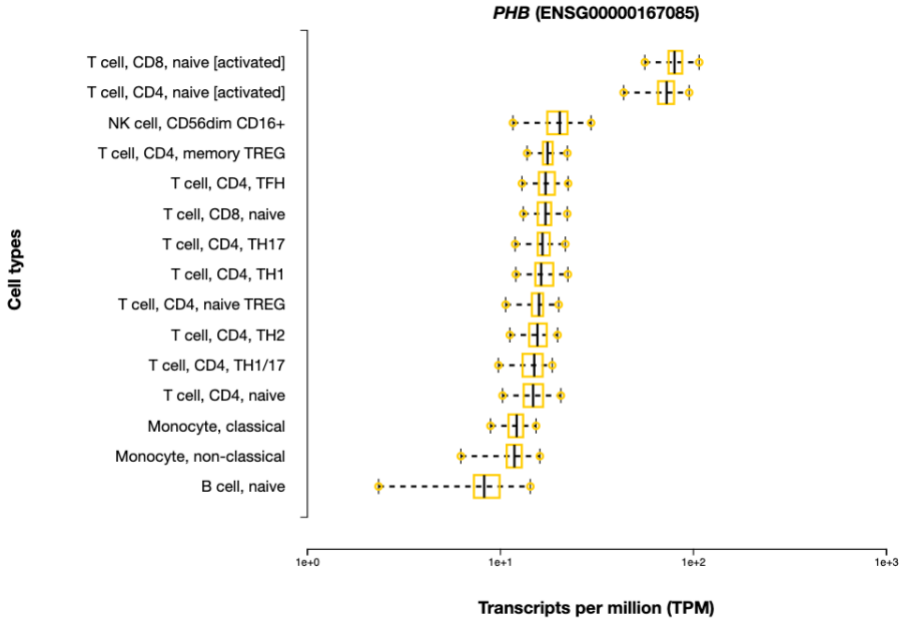
1.6 PHB and The Immune System

PHB has been associated with the immune system in many capacities. Around the same time PHB was discovered for its anti-proliferative property, PHB and PHB2 were separately shown to co-precipitate with the IgM receptor; this led to their alternative names, B cell receptor-associated protein of 32 kDa (BAP32) and BAP37 for PHB and PHB2, respectively [70]. Later, binding of the PHB complex to CD86 was found after B cell priming with CD40. The CD86-induced phosphorylation of phospholipase Cγ2, protein kinase Cα/βII and IκBα, and the IgG1 production all required CD86 binding to both PHBs [5883]. In primary human T cells, the mitochondrial PHB/PHB2 complex is upregulated and phosphorylated upon stimulation by CD3 and CD28, which were crucial steps to ensure T cell activation and viability [84]. Others have also demonstrated that PHB, under the influence of gonadotropin-releasing hormone, may be involved in the maturation of T cells during thymic growth [85]. When T cells are activated by inflammatory factors, phorbol myristate acetate (PMA) and ionomycin, expressions of PHBs are enhanced on the plasma membrane and co-localized with CD3 [67]. The PHBs act as a counter receptor for Siglec9, an adhesion molecule found on dendritic cells and macrophages, and inhibit ERK1/2 phosphorylation and T cell receptor signalling [86]. In T helper 17 cells, inhibition of the highly expressed surface PHB complex inhibited the CRAF/MAPK pathway, reduced IL-17 expression and increased $FOXP3^{+}$-expressing T regulatory cells in a multiple sclerosis animal model [68]. Figure 1.3 summarizes the RNA expressions of both PHB and PHB2 in immune cells. Notably, PHB is most highly expressed in T cells, followed by natural killer (NK) cells, monocytes, and B cells. PHB is also involved in platelet aggregation. Both PHBs are detected on the surface membrane lipid rafts of human platelets and interact with the protease-activated receptor 1, which initiates the platelet aggregation [87].

Diseases with an inflammatory component such as diabetes, cancer, and autoimmune diseases often present altered PHB expression, localization and/or function [88–90]. In both ulcerative colitis and Crohn's disease, PHB protein expression is decreased [91,92]. Sustained expression of PHB in intestinal epithelial cells reduced nuclear translocation of NFκB p65, NFκB/DNA binding, and the NFκB-mediated transcriptional activation of inflammatory factors [93]. Another study further showed that mice with intestinal epithelial cell-specific PHB overexpression have decreased colitis-associated tumour susceptibility [94]. In hepatocellular carcinoma patients, an increased level of IL-8 correlates with a significant reduction in survival. *In vitro* data suggest that PHB silencing preceded IL-8 mRNA upregulation through activation of NFκB p65 and AP1 [88]. Even though PHB was discovered and supported for its antiproliferative property, there are conflicting evidence regarding the role of PHB in tumorigenesis. PHB is found to be overexpressed in many tumour models and a predictor of poor prognosis, including gall bladder cancer, squamous cell lung cancer, and hepatocellular carcinoma [95,96]. The significance of PHB in tumorigenesis has been reviewed and discussed elsewhere, and the controversial function may be better answered in a cell-specific context [97].

Membrane PHB is targeted by a number of microorganisms, such as *Salmonella enterica* Typhi [98], Hepatitis C virus [99], Enterovirus 71 [100], Chikungunya virus [101], Human immunodeficiency virus [102], Dengue virus 3 [103], Herpes simplex virus 1 [104] and Japanese encephalitis virus [105]. The capsular polysaccharide of *Salmonella enterica* Typhi, V_i, interacts with the PHB complex on the surface lipid raft of intestinal epithelial cells [98]. In particular, V_i targets the MAPK pathway and inhibits the production of inflammatory cytokines from mononuclear phagocytes [98]. Furthermore, the Hepatitis C virus hijacks hepatocytes during viral pathogenesis through the upregulation of mitochondrial PHB. Interaction between PHB and the Hepatitis C virus core protein disrupts

cytochrome c oxidase and oxidative balance, leading to cellular oxidative stress [106]. Both Chikungunya virus and enterovirus 71 target surface PHB for internalization, while mitochondrial PHB is abducted for viral replication in enterovirus 71 [100,101].



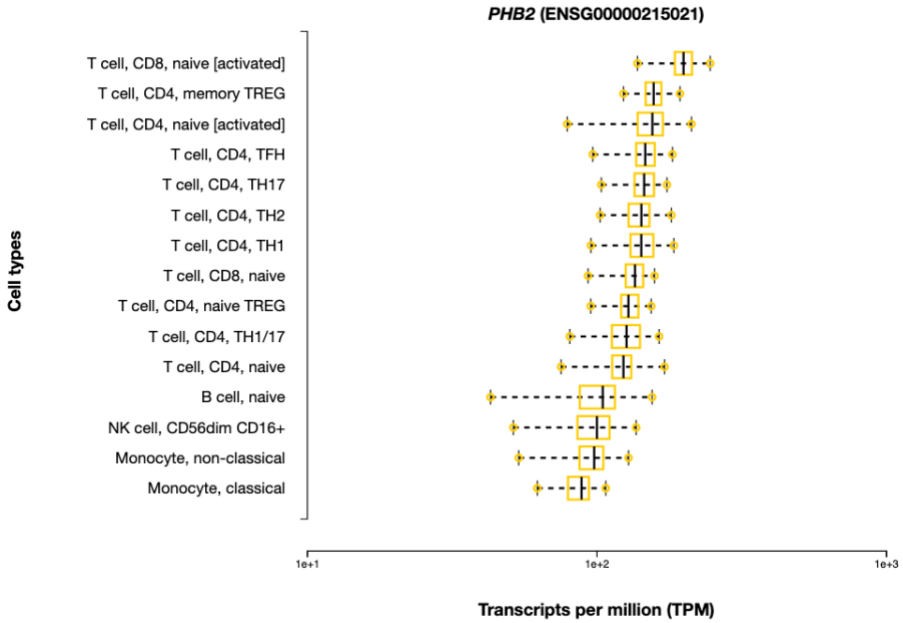


Figure 1.3. Human PHB and PHB2 expressions in immune cells. Images were taken from the Database of Immune Cell EQTLs, Expression, Epigenomics (DICE, https://dice-database.org/landing) under Explore gene and bulk RNA seq. Expression levels of each gene are expressed in transcripts per million (TPM) within the cohort of donor studies. Cell types are sorted according to median expression level within the cohort from highest to lowest. Boxes indicate 25% to 75% interquartile ranges, and whiskers indicate minimum and maximum. The graph is expressed on a log scale including both sexes [107].

1.6.1 PHB in the Macrophage

Very little is known about PHB in macrophages since the beginning of my Ph.D. thesis in 2017. One phagosome proteomics study identified PHB as a membrane protein in the phagosome with potential involvement in the lipid rafts [108]. This wasn't a surprise since other members of the SPFH superfamily are commonly found in the lipid raft of cellular membranes. Of note, lipid rafts play diverse roles in the cell, one of which is to compartmentalize cellular functions within the plane of the biological membranes [109]. This property provides a distinct environment for signalling molecules and receptors to efficiently regulate downstream pathways. The coalescing of lipid raft microdomains brings into proximity a new repertoire of protein-protein and protein-lipid interactions. This study also demonstrates that the identification of PHB in the phagosome is unlikely due to contamination, but rather due to association with membrane receptors at the cell surface [108]. Moreover, since microorganisms target PHB for entry, I speculate that PHB could serve as a signal to initiate phagocytosis.

In 2020, a study performed on RAW 264.7 macrophage cell line found decreased expressions of PHB mRNA and protein under LPS stimulation [110]. PHB knockdown systemically reduced both pro- and anti-inflammatory cytokines and markedly increased the NFκB activity [110]. These findings corroborated my experimental results in Chapter 5 that PHB is an early player in the macrophage polarization pathway and affects expressions of TNFα, IL-1β, IL-6, and IL-10.

Osteoclasts are specialized resident macrophages derived from hematopoietic stem cells; their differentiation and survival depend on the expression of the receptor activator of nuclear factor kappa B ligand (RANKL) [111]. One research group found that RANKL signalling is inhibited by overexpression of PHB, which inhibits the mature osteoclast formation [112]. Therapeutic targeting of PHBs has also been shown to inhibit osteoclastogenesis, which in turn improves osteoporosis [113]. While no subcellular location of PHB was mentioned in the study, it's implied that PHB is likely found on the plasma membrane.

1.7 Immunometabolism

Immunometabolism is an area of integrated research that studies the interplay and interdependence between the immune system and the metabolic pathways at the cellular and systemic levels. The two distinct fields of research have crossed paths owing to their inseparable connection in chronic diseases like obesity, inflammation, diabetes, and cancer. Adipose tissue, the liver and the hematopoietic system share a developmental heritage in primitive organisms, such as the fat body in *Drosophila* [114,115]. This shared heritage implies that there are molecules that integrate multiple pathways and crosstalk between tissues to achieve coordinated metabolic homeostasis. In higher organisms, these organs become specialized and distinct functional units. The immune system is recognized as an active participant in the endocrine and metabolic systems. Immune cells encounter diverse microenvironments under healthy and disease states that require different biochemical pathways, including glycolysis, the tricarboxylic acid cycle, oxidative phosphorylation, pentose phosphate pathway, fatty acid oxidation, and amino acid metabolism. On the other hand, metabolites produced by the endocrine organs greatly impact the development and activities of immune cells. In the case of obesity, hypertrophic adipocytes that exceed their carrying

capacity for triglycerides become dysfunctional and necrotic. Proinflammatory cytokines spewed out from these adipocytes, including TNFα, IL-1 and IL-6, and immunological factors, such as monocyte chemoattractant protein 1 (MCP1) and C-X-C motif chemokine ligand 1 (CCL1), attract resident macrophages and circulating monocytes, which initiate the local inflammatory response [116]. This untreated local inflammation in conjunction with oxidative stress and mitochondrial dysfunction within adipocytes produces a positive feedback loop. The result is a more chronic and systemic inflammation, which acts as a major culprit of metabolism disorders.

Since the start of my Ph.D. in 2017, this emerging field has been extensively reviewed from the perspective of evolution [117], pathogen growth and containment [118], infection [119], autoimmune-related diseases [120,121], cancers [122] and therapeutics [123,124] (such as single-cell application [125]). New findings are coming out regularly on harnessing the immune system's full potential to understand and treat chronic metabolic illnesses.

1.8 Macrophages

Macrophages were discovered more than 100 years ago by Ilya Metchnikoff [126]. By introducing a rose thorn into a starfish larva, he observed a cellular defence mechanism he called phagocytosis, which won him the Nobel Prize for Medicine in 1908. Macrophages are thus named after their phagocytic ability: Greek “macros”, large; “phagein” to eat. As we now know, they perform vital roles that go beyond this function in both immunity and tissue homeostasis, ranging from antigen processing and presentation during adaptive immunity to facilitating inflammatory response and tissue repair.

In immunology, macrophages were first classified under the mononuclear phagocytic system (MPS, earlier known as the reticuloendothelial system) introduced in 1969 [127]. Cells in the

MPS, including macrophages, dendritic cells, and monocytes, share similar morphology (mononucleated) and function [128,129]. Within this context, macrophages are thought to develop solely from circulating monocytes as they extravasate and play a central role in tissue homeostasis and innate immunity [130]. Dendritic cells are taunted as the most efficient antigen-presenting cells due to a reduced phagosomal degradative/cytotoxic ability and enhanced antigen presentation skill [131]. Monocytes are the major circulating phagocytes and serve as precursors to both macrophages and dendritic cells [130].

Later studies challenged the MPS categorization with evidence that these cells exhibit a unique ontogenetic trajectory and diverge from the common myeloid progenitors (CMP). For one, dendritic cells can arise from both monocytes and common dendritic cell progenitors (CDP); the latter is then subdivided into classical and plasmacytoid dendritic cells [132]. Macrophages may be seeded *in utero* from the yolk sac or fetal liver progenitors [133]. They acquire unique transcriptional profiles and functional capabilities depending on the resident tissue type and rely mostly on self-renewal [134]. Resident macrophages serve the purpose of site-specific and metabolite-specific regulation to maintain tissue homeostasis [135]. Other macrophage phenotypes are also observed under pathological conditions and given unique names. Table 1.1 lists the names, locations, and associated pathologies of known site-specific macrophages. During infection and inflammation, both *in situ* proliferation and diapedesis of circulating monocytes are possible routes to increase the macrophage population [136,137].

Table 1.1. Names, locations, and associated pathologies of known site-specific macrophages.

Name(s)	Location	Associated Pathologies
Adipose tissue macrophage	Adipose tissue	Obesity, insulin resistance, type 2 diabetes mellitus [138]
Alveolar macrophage Interstitial macrophage	Lung	Chronic obstructive pulmonary disease, pulmonary alveolar proteinosis [139]
Foam cell	Fatty plaque located on blood vessel walls	Atherosclerosis, ischemia, embolism, stroke, myocardial infarction, and other cardiovascular diseases [140]
Hofbauer cell	Placenta	Villitis, preterm delivery [141]
Kupffer cell	Liver	Alcoholic and non-alcoholic fatty liver diseases, hepatitis, hepatic fibrosis, steatohepatitis [142,143]
Langerhans cell	Skin	Langerhans cell histiocytosis, defects in wound healing [144]
Mammary gland macrophage	Breast	Breast cancer [145]
Metallophilic macrophage Marginal zone macrophage Red Pulp macrophage	Spleen	Autoimmunity, blood-borne infection [146,147]
Microglia	Brain	Alzheimer's disease, Parkinson's disease, frontotemporal dementia, other neurodegeneration and psychiatric disorders [148,149]
Muscularis gut macrophage Intestinal lamina propria macrophage	Gastrointestinal tract	Ulcerative colitis, Crohn's disease [150]
Osteoclast	Bone	Osteopetrosis, osteoporosis, Paget's disease, rheumatoid arthritis, bone tumours [151,152]
Pancreatic macrophage	Pancreas	Pancreatitis, pancreatic ductal adenocarcinoma [103, 153]
Peritoneal macrophage	Peritoneum	Infertility, peritonitis, endometriosis, and peritoneal carcinomatosis [154,155]
Renal macrophage	Kidney	Acute kidney injury, chronic kidney diseases, kidney fibrosis [156]
Tumour-associated macrophage	Tumour	Cancers, metastasis [157]

1.8.1 Macrophage Polarization

Macrophage polarization describes the activation of naïve macrophage (M0) and transition into a specific phenotype at a given space and time [158]. In keeping with the T cell subtyping, activated macrophages are broadly characterized as classically activated/proinflammatory (M1) or alternatively activated/anti-inflammatory (M2) based on stimuli, cytokine production, and metabolic programming [159]. Although considered a simplification, the M1/M2 dichotomy can be robustly defined by a set of unique mechanistic players involved in each pathway [160]. When properly controlled, the balance between these two polarized states determines the outcome of tissue response. However, when the balance is disrupted, pathophysiological conditions can occur. For example, when M1 macrophage activation becomes excessive, host tissue damage and chronic inflammation may ensue. On the other hand, the M2 macrophages are exploited by cancer cells to promote immune suppression and pro-healing mechanisms, thus allowing them to escape the host immune response [161]. For these properties, macrophages have been intensely studied for potential therapeutic strategies in many diseased states, where one predominant activated state is observed.

1.8.2 Classically-activated Macrophage, M1

The classically-activated M1 phenotype is associated with the helper T cell subtype 1 (Th1)-mediated immunity. Th1 response is best suited for the destruction of cells infected with intracellular pathogens. Similarly, the M1 response upregulates local and systemic inflammation, which enhances microbicidal and tumoricidal activities [162]. The M1 stimuli include pathogen-associated molecular patterns such as LPS, and proinflammatory factors such as TNFα, IFNγ, or a combination, which yield variations of a similar phenotype. *In vitro* experiments have also used granulocyte/macrophage colony-stimulating factor (GM-CSF) for M1 polarization. Major

cytokines produced from the M1 phenotype are TNFα, IFNγ, IL-1α and -1β, IL-6, IL-8, IL-12, IL-23; chemokines: CCL2/MCP1, CCL15, CCL20, CXCL9, CXCL10, CXCL11; superoxide anions, oxygen radicals and nitrogen radicals [163,164] [Figure 1.4].

IFNγ, also known as the macrophage-activating factor, was the first cytokine identified to induce M1 polarization [165]. IFNγ is produced by both innate and adaptive immune cells in response to stress and infection [163]. The binding of IFNγ to its receptor on macrophages triggers the Janus kinases (JAK) signal transducers and activators of the transcription (STAT) pathway. Tyrosine phosphorylation of JAK1/2 leads to phosphorylation and dimerization of STAT1, which translocates to the nucleus for transcriptional function [166]. Another branch of JAK1/2 signalling leads to the activation of the PI3K/Akt/mTOR pathway, which promotes effector protein production [166]. On the other hand, LPS-mediated M1 polarization signals through the adaptor molecule myeloid differentiation primary-response gene 88 (MyD88), which activates the NFκB pathway [167]. The NFκB pathway controls an array of inflammatory cytokine genes, including TNFα and IFNα/β, both of which augment M1 macrophage activation in an autocrine manner [166]. Despite being highly proliferative, M1 macrophages undergo a significant metabolic shift towards glucose metabolism and enhanced glycolysis under well-oxygenated conditions [168]. This aerobic glycolysis, termed the Warburg effect, has first been observed in cancer cells and later in non-cancerous proliferating cells. The rapid onset of glycolysis makes up for its low net yield of 2 ATP per glucose. This dependence on glycolysis is also seen in NK cells, activated B cells and effector T helper-1 and -17 cells, where glycolysis is required for proper differentiation and immune effector functions [169]. In addition to heavy dependence on glycolysis, there are three major areas of interest in the M1 energetic pathways. First, M1 macrophages exhibit an increased flux into the pentose phosphate pathway (PPP), which branches from the glycolysis intermediate, glucose-6-

phosphate (G6P) [Figure 1.4]. The oxidation of G6P in macrophages leads to the generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which lays the foundation for ROS production and bactericidal activity [170]. Second, the tricarboxylic acid (TCA) cycle in M1 macrophages is disrupted downstream of citrate and succinate, leading to the accumulation of these intermediates [171] [Figure 1.4]. Citrate accumulation and transport to the cytoplasm are important for the production of fatty acids and membrane biosynthesis. Cytosolic citrate is a substrate of ATP citrate lyase, producing acetyl-CoA and oxaloacetate, which are important in NO, ROS, and prostaglandin E2 (PGE2) production [171]. In addition, a breakage at citrate in the TCA cycle also promotes a shift toward itaconate generation [170]. Itaconate is immunomodulatory by activating the nuclear factor erythroid 2-related factor 2 (Nrf2) to limit inflammation [172]. Itaconate is also a direct inhibitor of succinate dehydrogenase (SDH), which further contributes to succinate accumulation in the mitochondria [170]. Succinate exhibits a pro-inflammatory effect through the activation of HIF1α and subsequently leads to IL-1β production [173]. Mitochondrial oxidative phosphorylation (OXPHOS) is comparatively downregulated in M1 macrophages [170]. This may be due to the nitrosylation of the electron transport chain, which inhibits mitochondrial respiration progressively. Conversely, some researchers demonstrated that stimulation of selective TLRs can increase both glycolysis and OXPHOS, which suggests the complexity of metabolic rewiring [174,175]. The glycolytic shift of M1 macrophages has been attributed to Akt signalling. Activation of Akt can directly increase glycolytic enzymes, promote mTOR complex 1 activity, and upregulation HIF-1α.

M1 macrophages are important players in host defence; but their actions are nonspecific, which produce tissue injuries when not tightly controlled. Indeed, M1 macrophages are mediators of autoimmune diseases and sterile chronic inflammation. Rheumatoid arthritis and insulin

resistance are prime examples of dysregulated M1 macrophages that sustain the pro-inflammatory environment and disease activity.

1.8.3 Alternatively activated Macrophage, M2

The alternatively activated M2 macrophages are initially associated with Th2-mediated immunity. It can be induced by a wide range of agents, including interleukins (IL-4, IL-10, IL-13, IL-33) glucocorticoids, TGFβ, and immune complexes. For this reason, M2 macrophages were further subcategorized into M2a, M2b and M2c [176]. M2 macrophages perform diverse functions and under certain conditions (M2a) counteract the classical activation pathway. The overall response centres on immunoregulation, resolution of inflammation, wound repair, angiogenesis, and resistance to parasites. M2 macrophages can be identified by their surface expression of CD80/86, CD163, mannose receptor (CD206), and MHCII. Major factors produced by the M2 macrophages are TGFβ, IL-10, insulin-like growth factor 1 (IGF1), resistin-like molecule alpha (RELMα/FIZZ1), chitinase-like 3 (Ym1/2), L-arginase 1, vascular endothelial growth factor (VEGF), 12,15-lipoxygenase, and chemokines: CCL1, CCL17, CCL18, and CCL22 [163,164,176,177] [Figure 1.4]. Fetal and adult resident macrophages are thought to display an M2 phenotype to maintain tissue homeostasis [178,179].

IL-4 is considered a hallmark stimulus of M2 polarization. Major sources of IL-4 come from basophils, mast cells, Th2 cells and M2 macrophages themselves. Following IL-4 stimulation, JAK1 auto- and cross-phosphorylate, which leads to STAT6 phosphorylation at its tyrosine residues. Subsequently, STAT6 homodimerizes and translocates to the nucleus as a transcription factor. Together with its coactivator, peroxisome proliferator-activated receptor PPARγ coactivator 1 beta (PGC1β), IL-4 activates multiple signalling pathways, including PI3K/Akt,

ERK/mTOR and PKC [180]. IL-13 produces similar effects on macrophages as IL-4 because they share a common receptor chain [177].

M2 macrophages have a more balanced metabolic profile and rely mainly on OXPHOS and mitochondrial respiration for the sustained production of energy [162]. IL-4 stimulation inhibits the mTOR1 complex but upregulates 5'AMP-activated protein kinase (AMPK) signalling. The TCA cycle is important to produce ATP and support the various biosynthetic pathways [Figure 1.4]. In M2 macrophages, it is mainly replenished at two entries: Acetyl-CoA and α-ketoglutarate. Acetyl-CoA comes from both glycolysis and beta-oxidation. Glutamine metabolism plays a major role in M2 macrophages: It replenishes α-ketoglutarate and contributes to the production of glutathione and hexosamine. Hexosamine is the precursor for UDP-GlcNAc, a high-energy donor for *O*-GlcNAc transferase that catalyzes the addition of *O*-GlcNAc to serine/threonine residues in the post-translational modification of proteins. In a fashion analogous to phosphorylation, *O*-GlcNAcylation has a great influence on cell adhesion and signalling events, and subsequently pathogen recognition [181]. The most distinguished change in M2 macrophages is the expression of arginase, where L-arginine is converted to ornithine and then polyamines for wound repair and cell proliferation [182].

The immunomodulatory role of M2 macrophages inadvertently provides an ideal condition for pathologies such as fibrosis and cancer progression. In allergic asthma, M2 macrophages facilitate myofibroblast differentiation and extracellular matrix deposition thereby increasing airway remodelling [183]. In an established tumour, tumour-associated macrophages (sometimes known as M2d) have immune-suppressive functions and produce growth factors that contribute to hypoxia-induced angiogenesis and tumour metastasis [184]. The primary focus of the thesis will be the M2a, which will serve as the representative M2 phenotype hereafter.

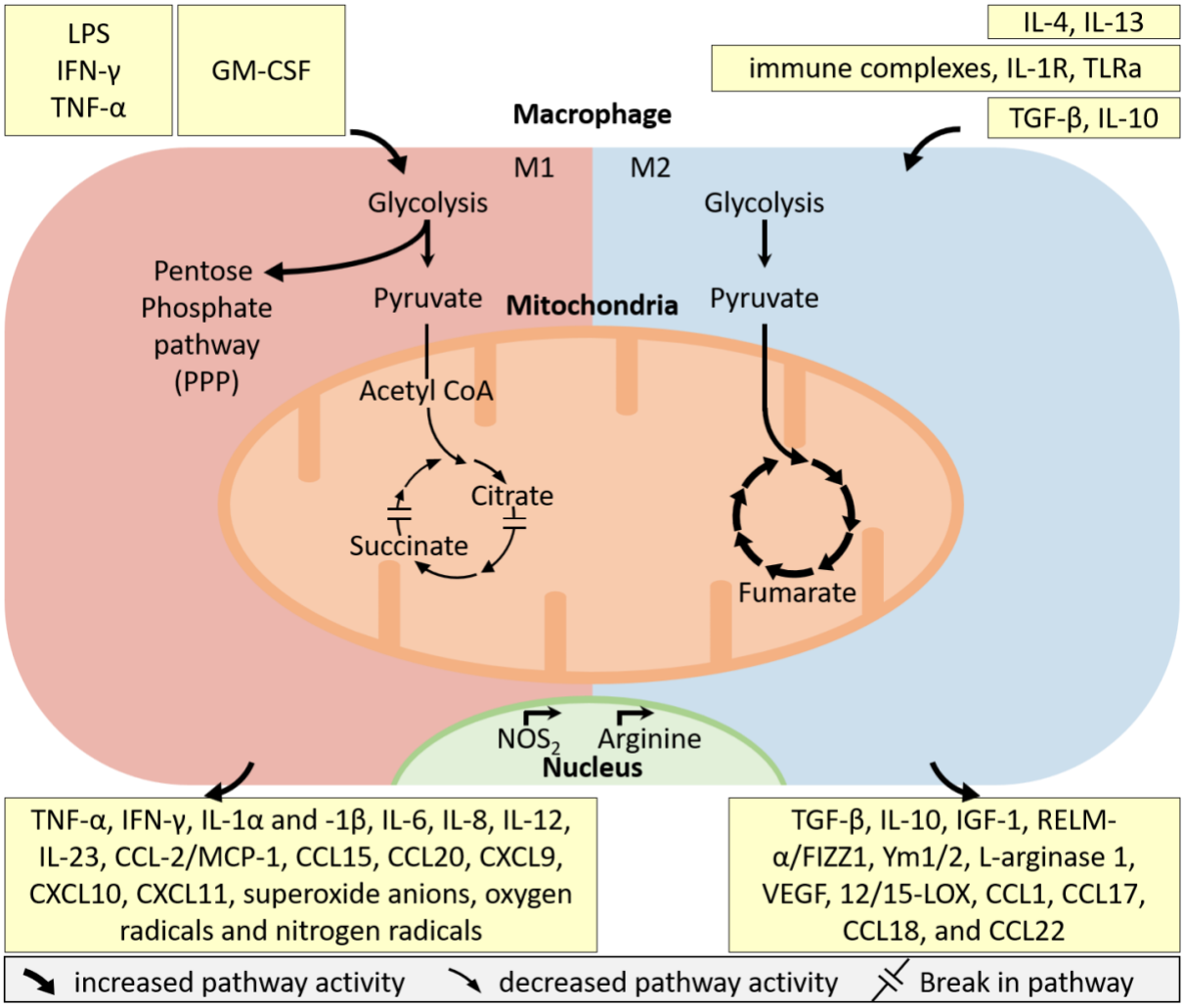


Figure 1.4. Macrophage polarization to the M1 and M2 phenotypes. M1 macrophages are induced by LPS, TNFα, IFNγ, or granulocyte/macrophage colony-stimulating factor (GM-CSF) *in vitro.* The M1 phenotype is associated with an increase in the utilization of the glycolysis and pentose phosphate pathway (left). Conversely, these cells demonstrate a reduced activity of mitochondrial respiration with the TCA cycle being broken at two points: after citrate and after succinate. Alternatively, M2 macrophages are more plastic and can be induced by a variety of factors, including IL-4/IL-13, IL-10, IL-13, IL-33) glucocorticoids, TGFβ, and immune complexes to generate different functional outcomes. Specifically, the M2 phenotype of interest (induced by IL-4) is characterized by increased fatty acid oxidation and mitochondrial respiratory chain activity.

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CHAPTER 2: RATIONALE, HYPOTHESIS, AND OBJECTIVES

2.1 Rationale

Prohibitin (PHB) has clear and distinct functions within different subcellular compartments. In the mitochondria, PHB behaves as a chaperone and facilitates mitochondrial biogenesis and integrity. This function is most noticeable in adipocytes, where enhanced expression of PHB leads to increased adipogenesis [1,2]. On the plasma membrane, PHB acts as an adaptor protein and participates in cell signalling through post-translational modifications and associated interacting partners [3–5]. Specifically, phosphorylation at Tyr-114 has been deemed an important negative regulator in insulin signalling facilitated through its interaction with tyrosine phosphatase, Shp1 [6,7]. Evidence also suggest that Tyr-114 phosphorylation has a role in mast cell activation and allergic response, where phosphorylated PHB facilitates the recruitment of cytoplasmic tyrosine kinases, Fyn, and Lyn [8]. Diseases with an inflammatory component such as diabetes, cancers, and autoimmune diseases often present altered PHB expression, localization, and/or function [9]. PHB in diverse model systems is correlated to the level of ROS and inflammatory pathologies [10–12]. Specifically, overexpression of PHB in intestinal epithelial cells has been shown to attenuate colonic inflammation in experimental models of colitis, whereas its deficiency promotes inflammation and increases sensitivity to liver injury [13]. While it is evident that PHB is intimately involved in the functioning of immune cells, its specific role in macrophages remains unclear. My research question on this topic stemmed from two transgenic mouse models developed in our laboratory: Mito-Ob and mMito-Ob, overexpressing PHB and Y114F under the *Fabp4 (aP2*) gene promoter, respectively [14,15]. The *Fabp4* gene promoter is expressed in adipocytes and monocytic cell lineage, which allowed us to spontaneously manipulate both cell types [16,17]. Upon phenotypic

characterizations, it was discovered that the Mito-Ob and mMito-Ob mice shared metabolic phenotypes, which included sex-neutral obesity but male-specific metabolic dysregulations [14,15]. These observations suggested that the Y114F mutation did not affect the development of obesity and its associated metabolic outcomes. However, Mito-Ob and mMito-Ob mice exhibited distinct immunophenotypes, in which male mMito-Ob mice developed lymph node tumours showing increased proliferation of macrophages and splenomegaly [15]. A summary of the pathophysiological changes observed in the two transgenic mouse models is presented in Table 2.1. Further analyses of tissue samples from the male Mito-Ob and mMito-Ob mice showed changes in the expression level of immune checkpoint components, T cell population, and serum markers of immune dysregulation. These results suggest that the Tyr-114 phosphorylation site in PHB is important in promoting the observed immunophenotypes.

Table 2.1. Genotypes and phenotypes of female and male Mito-Ob and mutant Mito-Ob transgenic mouse models compared to wild-type [14,15].

Males	**Wild-type**	**Mito-Ob**	**mMito-Ob**
Genotypes	CD-1 control	PHB gene under aP2 promotor	Y11F-PHB gene under aP2 promotor
Phenotypes	Metabolic Control	• Obese • Glucose intolerance • Insulin resistance • Liver tumour	• Obese • Lymph node tumours • Splenomegaly
Females	**Wild-type**	**Mito-Ob**	**mMito-Ob**
Genotypes	CD-1 control	PHB gene under aP2 promotor	Y11F-PHB gene under aP2 promotor
Phenotypes	Metabolic Control	• Obese • Normal immune functions	• Obese • Normal immune functions

2.2 Hypothesis and Objectives

PHB is an essential protein with multiple subcellular localizations and cell- and tissue-specific functions. Based on available evidence of PHB in macrophages and other immune cell types, I hypothesize that PHB plays a key role in the regulation of macrophage functional plasticity (as measured by M1/M2 polarization) through cell signalling and metabolic output.

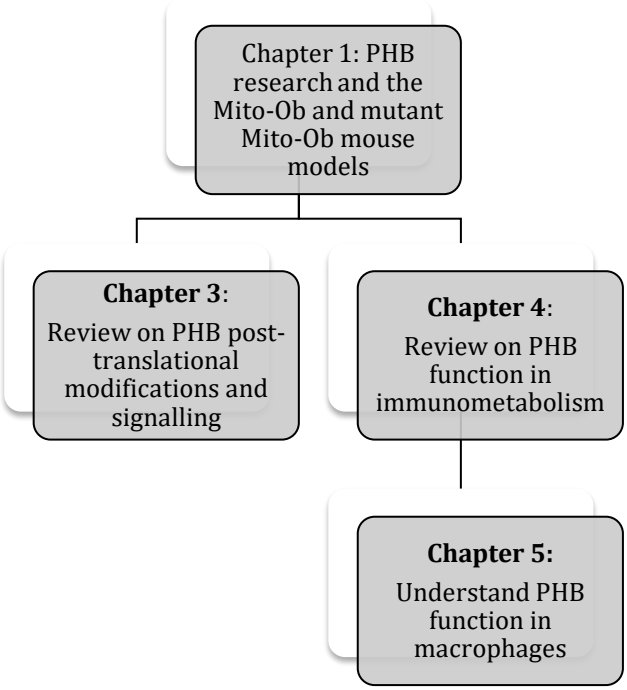

Figure 2.1. Thesis overview.

The hypothesis will be addressed through the following objectives:

Objective 1. Determine the role of PHB phosphorylation sites (especially Tyr-114) in immune cell signalling.

Objective 2. Determine the subcellular localization and expression level of PHB in macrophages.

Objective 3. Determine changes in signalling pathways during macrophage polarization upon PHB overexpression.

Objective 4. Determine changes in metabolic status and immunophenotypes upon PHB overexpression.

Objective 5. Determine changes in immune cell populations in transgenic mouse models, Mito-Ob and mMito-Ob.

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CHAPTER 3: PROHIBITIN: A POTENTIAL THERAPEUTIC TARGET IN TYROSINE KINASE SIGNALING.

Preface

The phenotypic difference between Mito-Ob and mMito-Ob mice sheds light on the importance of Tyr-114 phosphorylation in PHB-facilitated adipose-immune interaction and in the sex dimorphic role of PHB. Phosphorylation of PHB at Tyr-114 has been described in immune signalling in the past. It is also a key residue that negatively regulates tyrosine kinase signalling under insulin stimulation. A total of eight phosphorylation sites are found in PHB, most of which will be examined in terms of their mechanistic action in regulating different signalling pathways. The purpose of this narrative review is to examine PHB post-translational modifications with an emphasis on the phosphorylation of tyrosine. Prior to this, PHB tyrosine phosphorylation in different cells has not been collectively reviewed. In addition, the connection between PHB tyrosine phosphorylation and various signalling pathways, as well as the connection between these pathways due to PHB regulation, are not explored. In this chapter, we provided the context for examining PHB Tyr-114 and the background information on the relevance of PHB Tyr-114 function and the consequences of its dysregulation.

Contributions of Co-Authors:

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Yang Xin Zi Xu: manuscript preparation, figure preparation, manuscript revisions

Suresh Mishra: manuscript preparation, figure preparation, manuscript submission, manuscript revisions

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3.1 Abstract

Prohibitin (PHB) is a pleiotropic protein with roles in fundamental cellular processes such as cell proliferation and mitochondrial housekeeping, and in cell- or tissue-specific functions such as adipogenesis and immune cell functions. Different functions of PHB are mediated through its cell compartment-specific attributes, which include an adaptor molecule in membrane signalling, a scaffolding protein in mitochondria, and a transcriptional co-regulator in the nucleus. However, the precise relationship between its distinct cellular localization and diverse functions remains largely unknown. Accumulating evidence suggest that phosphorylation of PHB has roles in a number of cell signalling pathways and in its intracellular trafficking. Herein we discuss the known and potential importance of site-specific phosphorylation of PHB in regulating these features. Finally, we will discuss this in the context of new evidence from the tissue-specific transgenic mouse models of PHB including a mutant PHB lacking a crucial tyrosine phosphorylation site. We conclude with an opinion that PHB can be used as a potential target for tyrosine kinase signal transduction-targeted therapy including insulin, growth factors, and immune signalling pathways.

3.2 Introduction

Prohibitin (PHB or PHB1) was first identified as an anti-proliferative gene and its cDNA was isolated by differential hybridization to RNA from normal versus regenerating liver of rats [1]. *PHB* gene is present on chromosome 17q21 locus, a region associated with propensity to visceral fat deposition in humans [2]. Microinjection of *Phb* mRNA into normal human fibroblasts attenuated DNA synthesis and led to their growth inhibition [3]. Later, it was discovered that growth inhibition caused by *Phb* mRNA was due to the 3' untranslated region and not the coding region [4]. PHB protein is a member of a highly conserved family of proteins known as the Band-7 or prohibitin

domain family that includes the repressor of estrogen activity (REA or PHB2), stomatins, HflK/C, flotillins, and plant defence protein family known as hypersensitive-induced reaction (HIR) [5]. There are three major domains in the protein sequence of PHB: The N-terminal hydrophobic alpha-helix containing a membrane-anchoring domain, the mid-region containing the PHB domain, and the C-terminus containing a coiled-coil domain with a nuclear localization sequence [6]. The role of PHB has been implicated in diverse fundamental cellular functions such as cell proliferation [7], cell cycle control [8], differentiation [9], protection from oxidative stress [10], and it also acts as a molecular chaperone [11]. PHB is a ubiquitously expressed protein present in various cellular compartments such as the cell membrane [12], mitochondria [7,13] and the nucleus [14]. Though PHB was discovered more than 25 years ago, there is little known with regard to the regulation of this protein. Unfortunately, the global knockdown of *Phb* leads to embryonic lethality in multicellular organisms such as *C. elegans* and mice, thus preventing researchers from identifying the molecular functions of PHB at an organismic level [15,16]. PHB is known to undergo various posttranslational modifications such as *O*-GlcNAc modification [17], palmitoylation [18], ubiquitination [19,20], phosphorylation [13,17,21–23] and cysteine oxidation [24].

Posttranslational modification plays an important role in the regulation, stability, trafficking, and cell signalling functions of proteins. Some of the commonly occurring posttranslational modifications in proteins are known to occur at the same residue in a mutually exclusive manner or regulate each other in the nearby residues. For example, *O*-GlcNAc modification and serine/threonine phosphorylation in certain proteins that occur at the same residues have been shown to be regulated by tyrosine phosphorylation in the nearby residues, and they influence each other [17]. Similarly, acetylation, ubiquitination and methylation may occur at the same lysine residue in a mutually exclusive manner and add diversity to protein functions and

their regulation [25]. Phosphorylation is the most extensively studied posttranslational modification, which orchestrates a variety of cellular and molecular events such as cell proliferation, differentiation, and cell death. It is estimated that more than 30% of cellular proteins contain covalently attached phosphate groups at a ratio of 1800:200:1 for serine, threonine and tyrosine, respectively [26]. Phosphorylation is important for normal cellular functions; dysregulated phosphorylation has been implicated in various disease conditions such as cancer, diabetes, and other neurological disorders. Phosphorylation is a fundamental posttranslational modification of PHB that regulates its function, intracellular trafficking, and binding specificity to certain proteins. There are two major forms of phosphorylation known to occur in PHB: Serine/threonine phosphorylation and tyrosine phosphorylation. In this review, we will highlight the roles of tyrosine phosphorylation in PHB. In the human PHB protein, there are only four tyrosine residues at positions 28, 114, 249 and 259, all of which are highly conserved among different species [27]. PHB is known to undergo tyrosine phosphorylation at multiple residues upon activation by various stimuli [17,21,22]. These stimuli include insulin, epidermal growth factor, platelet-derived growth factor and insulin-like growth factor [21,22,28]. Recent discoveries have pointed out a crucial role of PHB phosphorylation in the MEK/ERK pathway, PI3K/Akt pathway, TGFβ pathway and STAT3 pathway [21,29–31]. Herein, we describe the role of PHB tyrosine phosphorylation in various signalling pathways and its mechanism of action in regulating these signalling molecules.

3.3 Tyrosine phosphorylation of PHB and insulin signalling

Activation of the insulin signalling pathway proceeds with insulin receptor dimerization and auto-phosphorylation of its cytoplasmic domains [32]. These events lead to an increased tyrosine kinase activity of insulin receptor towards its substrates. Upon activation, the insulin receptor

complex phosphorylates insulin receptor substrates (IRS) and Src homologous/collagen proteins (Shc). These dynamic events lead to the activation of a cascade of signalling pathways, including the PI3K/Akt pathway and MEK/ERK pathway [32]. PI3K consists of two subunits: a catalytic subunit, p110 and a regulatory subunit, p85 [33]. PI3K promotes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 acts as a second messenger and activates the downstream target, alpha serine/threonine protein kinase (Akt, also known as protein kinase B). Activation of Akt leads to various biological effects including the suppression of apoptosis by inactivating the pro-apoptotic molecules such as BCL2-associated agonist of cell Death (BAD), BCL2-associated X (BAX) and caspase 9 [34]. The other arm of the tyrosine kinase signalling pathway is activated by the binding of IRS or Shc to the growth factor receptor-bound protein 2 (GRB2) and Sons of sevenless (SOS) [32]. These events lead to the activation of Ras and RAF1 signalling molecules, which eventually activate MEK and ERK1/2, and the translocation of ERK1/2 to the nucleus [35]. Upon translocating to the nucleus, ERK1/2 activate several mitogenic factors that lead to the survival and proliferation of cells.

Our laboratory has reported that phosphorylation of PHB at Tyr-114 residue has a role in attenuating insulin-signalling [21]. Initially, PHB is phosphorylated at Tyr-114 by the insulin receptor complex. Once phosphorylated, it creates a binding site for SH2 domain-containing protein tyrosine phosphatase 1 (Shp1); and through Shp1, PHB alters the phosphorylation of Akt and glycogen synthase kinase 3 beta (GSK3β) [21]. This finding suggests that insulin-induced tyrosine phosphorylation of PHB and subsequent recruitment of Shp1 may modulate IRS, PI3K activity and the downstream insulin signalling pathway. Furthermore, our laboratory has previously shown that PHB interacts with *O*-GlcNAc transferase (OGT) and becomes *O*-GlcNAc modified at Ser-121 and Thr-258 [17]. These two sites in PHB are in close proximity to two tyrosine

phosphorylation sites, Tyr-114 and Tyr-259 [17]. The substitution of Tyr-114 and Tyr-259 with phenylalanine led to decreased *O*-GlcNAc modification in PHB [17]; whereas, the substitution of Ser-121 and Thr-258 with alanine and isoleucine respectively led to increased tyrosine phosphorylation of PHB [17]. These findings suggest that there is a strong regulatory association between tyrosine phosphorylation and *O*-GlcNAc modification at Ser/Thr residues of PHB [Figure 3.2]. Although the biological significance of *O*-GlcNAc modification of PHB remains unclear, its relationship with functionally relevant phosphorylation sites in PHB (Tyr-114, Ser-121, Thr-258, and Tyr-259) indicates an important role in the regulation of PHB functions [17,36]. This binary switch between the two posttranslational modifications provides new mechanistic insight into various cell signalling pathways, which warrants further investigation.

PHB is also known to interact with PIP3 and attenuates insulin signalling [22]. A basic local alignment search tool (BLAST) analysis of the PHB protein sequence revealed that PHB contains conserved lipid-binding PX domains, which may help PHB in interacting with PIP3 and other lipids [22]. The Tyr-114 phosphorylation site is in close proximity to the putative PIP3 binding domain of PHB [22]. Thus, the phosphorylation status of Tyr-114 in PHB may influence other interacting partners of PHB, directly or indirectly, that has a role in PIP3 signalling. Several reports showed that PHB can translocate from mitochondria to the plasma membrane [18,37] and from the nucleus to mitochondria upon activation by various stimuli or agents [10,38,39]. But, the mechanisms involved in the intracellular trafficking of PHB are not well understood. Our laboratory have shown that PHB undergoes palmitoylation at Cys-69 and interacts with Eps 15 homology domain protein 2 (EHD2) [18]. This modification facilitates the translocation of PHB to the plasma membrane and the subsequent tyrosine phosphorylation of PHB [18]. It is the first report that elucidates the mechanism behind the translocation of PHB to the plasma membrane. Taken

together, these studies suggest that tyrosine phosphorylation of PHB plays an important role in modulating PI3K/Akt signalling pathway and has a relationship with other post-translational modifications [Figure 3.3]. Perturbation of tyrosine phosphorylation in PHB leads to impaired insulin signalling and altered cell metabolism, and these events eventually may lead to the development of type 2 diabetes and cancer. Hence, targeting PHB and its tyrosine phosphorylation may be novel therapeutic targets for the treatment of diseases involving dysregulation of the PI3K/Akt signalling pathway.

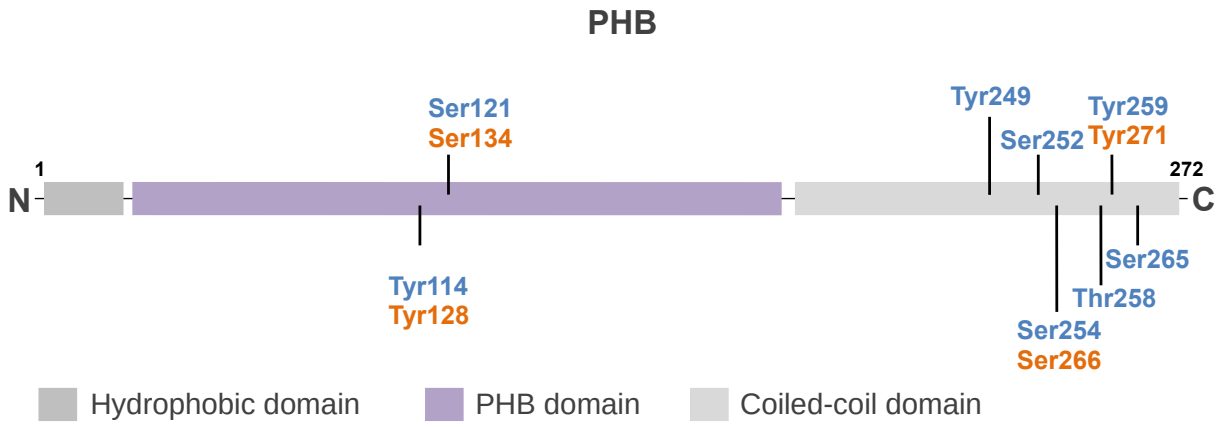

Figure 3.1. A schematic diagram showing the location of known phosphorylation sites in different structural domains of PHB. The conserved phosphorylation sites in PHB2 are shown in orange. Ser: Serine; Thr: Threonine; Tyr: Tyrosine.

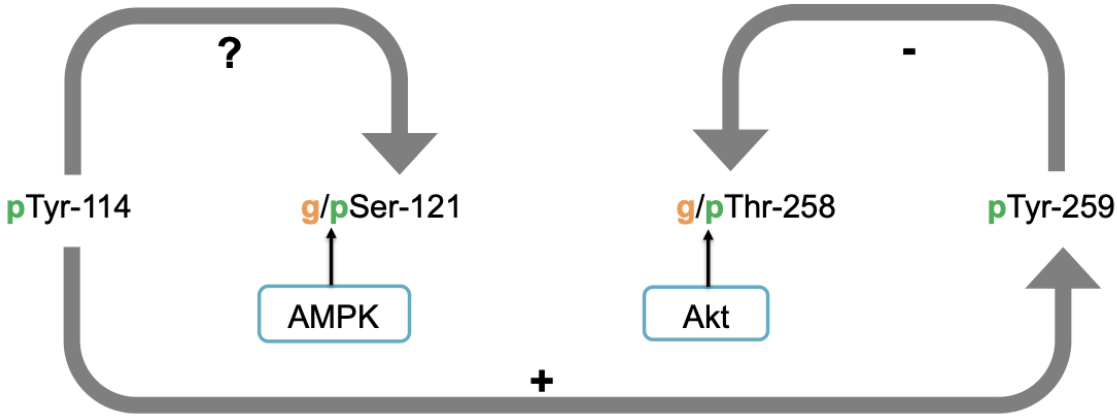

Figure 3.2. A schematic diagram showing known and potential crosstalk between tyrosine/serine/threonine phosphorylation and *O*-GlcNAc modification in PHB. Ser: Serine; Thr: Threonine; Tyr: Tyrosine; g: *O*-GlcNAc modified; p: Phosphorylated.

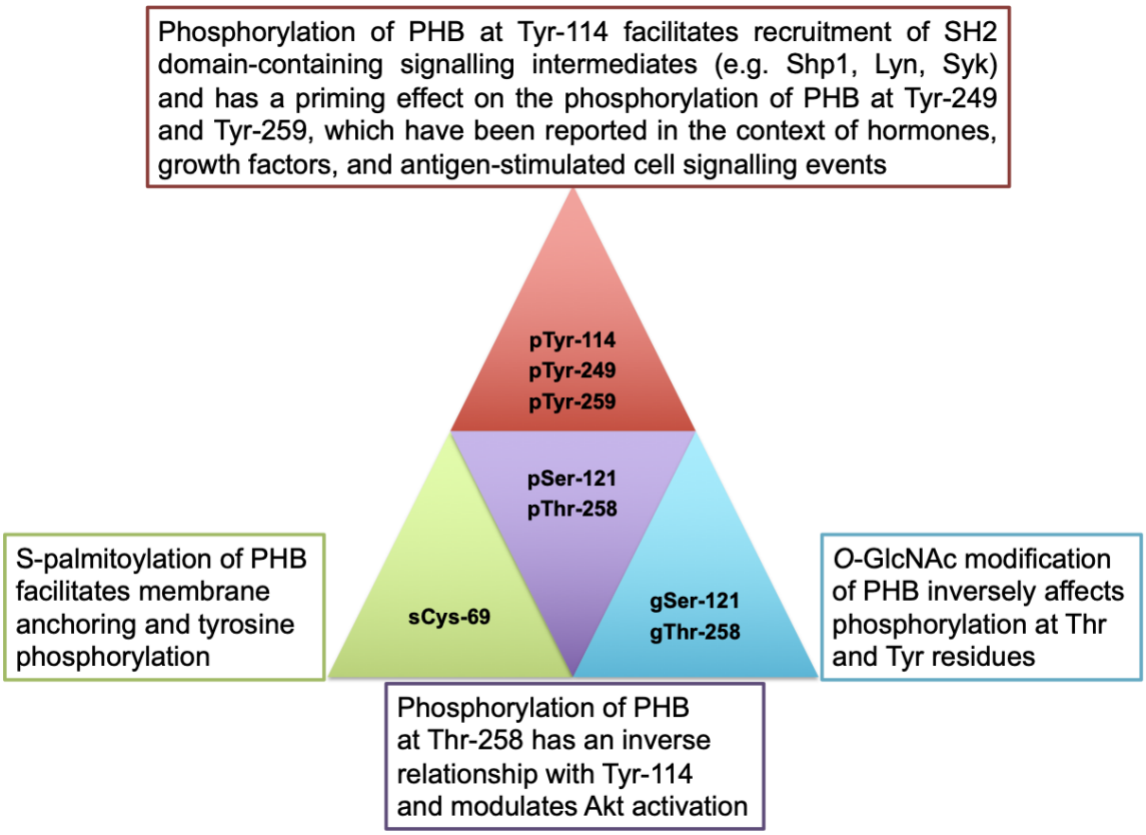

Figure 3.3. A schematic diagram showing the relationship among various post-translational modifications of PHB and their relevance in the regulation of PHB functions and cell signalling events [17]. Ser: Serine; Thr: Threonine; Tyr: Tyrosine; Cys: Cysteine; g: *O*-GlcNAc modified; p: Phosphorylated; s: S-palmitoylation.

3.4 Phosphorylation of PHB and Akt signalling

Akt is responsible for mediating various biological responses such as cell growth, cell differentiation, and apoptosis. It is a major signalling molecule downstream of PI3K [40]. For the activation of Akt, phosphorylation at Thr-308 is required; to attain its maximal activity, phosphorylation at Ser-473 is also required [41]. Dysregulation of Akt activity is known in various tumour malignancies and other diseases [42]. Interestingly, PHB has been identified as a target protein of Akt [40]. In human pancreatic cancer cells, PHB is shown to be phosphorylated by Akt [40]. In these cells, Akt-induced phosphorylation of PHB occurs at Thr-258, which is located within the Akt consensus motif R-x-R-x-x-S/T in PHB. Although Thr-258 is not conserved in PHB2, it has been reported that Akt can phosphorylate Ser-91 and Ser-176, which are present in Akt consensus motifs in PHB2 [43]. Phosphorylation of PHB by Akt can potentially impact various cellular functions of PHB and its stability. Our laboratory has shown that the phosphorylation of PHB at Thr-258 and Tyr-114 has opposing effects on insulin signalling [22]. The phosphorylation of PHB at Thr-258 upregulates insulin signalling, and its substitution by isoleucine results in the downregulation of both Thr-308 and Ser-473 phosphorylation in Akt [22]. Furthermore, the phosphorylation of PHB at Tyr-114 downregulates insulin signalling, and its substitution by phenylalanine results in the upregulation of Akt phosphorylation at Thr-308 and Ser-473 [22]. It is possible that the Akt phosphorylation of PHB at Thr-258 can impact its tyrosine phosphorylation at Tyr-114 and vice versa. Consistent with previous findings, a recent report has demonstrated that Akt phosphorylates PHB at Thr-258 in human bladder cancer cells [44]. Phosphorylation of PHB by Akt promotes its mitochondrial localization and leads to the proliferation of bladder cancer cells [44]. Furthermore, the substitution of Thr-258 by alanine induced cell death in bladder cancer cells

[44]. These results demonstrate that PHB is an important regulator during bladder cell tumorigenesis and that the phosphorylation of PHB at Thr-258 plays a key role in this process.

Furthermore, PHB has been shown to physically interact with RAF and activate RAS-induced RAF/MEK/ERK signalling upon epithelial growth factor (EGF) stimulation [29]. RAF1 is phosphorylation at Ser-259 under resting conditions; upon EGF stimulation, RAF1 is dephosphorylated at Ser-259 and subsequently phosphorylated at Ser-338 to become activated. In this context, it is important to note that Ser-259 in RAF1 is located in the Akt consensus motif R-x-R-x-x-S/T [45]. Therefore, it is possible that Thr-258 phosphorylation in PHB may compete with Ser-259 phosphorylation of RAF1 by Akt, thereby reducing the inhibition of RAF1 and facilitating its activation to promote cancer cell proliferation. In a recent report, researchers demonstrated that phosphorylation of PHB at Thr-258 and Tyr-259 correlated with the invasiveness of human cervical cancer cells, where a reduction in invasiveness was observed when phosphorylation was absent on both residues. Interestingly, the substitution of Tyr-259 for phenylalanine in PHB decreases the phosphorylation of Thr-258, but the substitution of Thr-258 for isoleucine has no effect on the phosphorylation of Tyr-259 [37]. The phosphorylation at Tyr-259 in PHB appears to have a unilateral regulatory role in the phosphorylation of Thr-258. Furthermore, the Thr-258 phospho-mutant of PHB prevents RAF1 activation indicating its crucial role in the induction of RAF1 activation [37]. It is anticipated that molecules targeting PHB and its phosphorylation on the plasma membrane may be effective in decreasing cancer cell metastasis and invasiveness. The above experimental findings highlight the crucial role played by PHB phosphorylation in cancer cells. However, more studies are required to determine the role of PHB and its phosphorylation by Akt at serine/threonine residues, as well as their relationship with tyrosine phosphorylation of PHB.

3.5 Tyrosine phosphorylation of PHB in T cell signalling

There are a number of reports that have identified PHB as a plasma membrane-associated protein in T cells and demonstrated its role in T cell receptor signalling [12,23,46]. Stimulation of mouse T cells with anti-CD3 and anti-CD28 antibodies remarkably induces the cell surface expression of PHB [12], which presumably participate in the T cell receptor-mediated signalling cascade. PHB has been shown to be an endogenous ligand for sialic acid binding lectin 9 (Siglec9) and negatively regulates T cell signalling [46]. Furthermore, *Salmonella typhi*, which causes typhoid in humans, targets PHB present on the T cells and downregulates T cell-mediated immune signalling [47,48]. In a separate study, to identify differentially expressed proteins upon activation of human primary T cells, the authors have found that the expression levels of *PHB* and *PHB2* mRNAs and proteins were highly upregulated upon T cell activation [23]. Using proteomic analysis, the authors have provided convincing evidence that PHB and PHB2 become phosphorylated as a phosphocomplex in the inner mitochondrial membrane of human primary T cells [23]. During this process, PHB is shown to be phosphorylated at serine residues and PHB2 is phosphorylated at both serine and tyrosine residues [23]. Furthermore, functional studies revealed that PHB/PHB2 phosphocomplex is required for the survival of differentiated T cells [23]. It has been shown that the phosphorylation of PHB2 at Tyr-248 is important for protein-protein interactions but not for the formation of the PHB/PHB2 complex [23]. Further analysis revealed that PHB/PHB2 phosphocomplex is required for mitochondrial homeostasis in T cells [23]. It is possible that phosphorylation of PHB/PHB2 at other tyrosine residues may also have a role in T cell maturation and warrants further investigations.

3.6 Tyrosine phosphorylation of PHB in mast cell signalling

Mast cells play a key role in the process of inflammation [49]. They are the major effector cells that drive allergic responses. Upon stimulation with antigens, mast cells secrete a large number of granules that contain histamine and other inflammatory mediators such as cytokines and eicosanoids [50,51]. In mast cells, antigen binds to the high-affinity IgE receptor (FcεRI) at the plasma membrane [50,51]. The presentation of antigen to IgE results in the aggregation of FcεRI complexes, and the rapid tyrosine phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) of the beta and gamma chains of FcεRI by the Src family of tyrosine kinases, such as Lyn [50,51]. Tyrosine phosphorylation of the gamma chain of FcεRI by Lyn leads to the recruitment of another cytoplasmic tyrosine kinase, Syk, to the gamma chain of FcεRI [50,51]. The activation of Syk is essential for the activation of important signalling molecules such as transmembrane adaptor protein linker for the activation of T cells (LAT), phospholipase C (PLC), Src homology 2 (SH2) domain-containing leukocyte protein 76 (SLP76) and Gab 2 [51]. The cascade of signalling events leads to the release of inflammatory markers that induces an allergic response.

It has been shown that PHB is abundant in the intracellular granule membrane of mast cells and has a critical role in antigen-stimulated allergic response [51]. Antigen binding to IgE receptors triggers the translocation of PHB from the granules to the plasma membrane and subsequent tyrosine phosphorylation of PHB by Lyn [51]. These experimental findings also suggest that PHB acts as a scaffold protein to facilitate the phosphorylation of the FcεRI gamma chain by Lyn [51]. Point mutation studies have revealed that phosphorylation at Tyr-114 and Tyr-259 is required for the recruitment of Syk to the FcεRI gamma complex and for the activation of mast cells [51]. These experimental findings highlight that the phosphorylation of PHB at Tyr-114 and Tyr-259 plays a crucial role in the association of PHB with the membrane, where it acts as a scaffold protein for

the FcεRI gamma and Syk complex during antigen-stimulated signalling events in mast cells. Similar to previous findings in 3T3-L1 and C2C12 cells from our laboratory [18], a Cys-69 mutant of PHB in mast cells was unable to translocate to the plasma membrane and failed to participate in the antigen-stimulated mast cell signalling [50]. Collectively, these studies suggest that both palmitoylation and phosphorylation of PHB are required for its translocation to the membrane and/or membrane association. More studies are required to understand the compartment-specific functions of PHB in different immune cell types.

3.7 Phosphorylation of PHB in B cell signalling

The association of PHB with the cell membrane of B cells was first discovered over 20 years ago [52]. PHB was shown to interact with the IgM receptor present on B cells [52]. However, the functional significance of the PHB-IgM association was not explored further until recently. It has now been shown that PHB/PHB2 complex and the cytoplasmic domain of CD86 cooperate in mediating CD86 signalling in B cells [53]. CD86 is a transmembrane glycoprotein associated with B cells, dendritic cells and macrophages [53,54]. In one study, Lucas *et al.* sought to identify potential signalling intermediates that associate with CD86, as well as their role in CD86 signalling pathway in B cells [53]. Under normal conditions, the expression level of CD86 was found to be low in resting B cells. Upon engagement with B cell receptor, CD40, or IL-4 receptor, the level of CD86 expression increases in B cells [55–58]. Unlike other transmembrane signalling molecules, CD86 contains a short cytoplasmic domain that is devoid of tyrosine phosphorylation motifs. By using a proteomic approach, the authors identified that in CD40/IL4-primed B cells, there is an interaction between PHB, PHB2 and CD86 [53]. CD86 engagement on the surface of the primed B cells activates two signalling pathways, which leads to the activation of NFκB [58]. Inhibition of these signalling

cascades in B cells decreases the expression of OCT2, which in turn affects the rate of IgG production [58]. Taken together, these results demonstrate that both the PHB/PHB2 complex and an intact CD86 cytoplasmic domain are required to mediate CD86 signalling. In these reports, however, the authors did not identify the residues that are phosphorylated in PHB. Based on the information discussed above, it is possible that the phosphorylation of PHB at tyrosine residues (Tyr-114 and Tyr-259) may be critical in CD86 signalling in primed B cells. Future point mutation studies can provide clues regarding the role of tyrosine phosphorylation of PHB in primed B cell signalling. In another report, Paris *et al.* have shown that Syk and PHB can interact with each other in a phosphorylation-dependent manner in antigen receptor-mediated B cell signalling [59]. It was found that PHB becomes phosphorylated at tyrosine residues and participates in immune signalling, but the specific phosphorylation site also remains to be identified [59]. As there are four tyrosine residues in PHB, it is likely that the phosphorylation occurs at previously known phosphorylation sites (Tyr-114, Tyr-249 and Tyr-259) in a number of cell types and tissues including immune cells [45,51]. In addition, whether the role of PHB in B cells requires intracellular trafficking remains unclear. More studies are required to understand the compartment-specific functions of PHB in cell signalling of different immune cell types. Moreover, a role for PHB in TGFβ and STAT3 signalling pathways has been reported [30,31]. However, the potential involvement of tyrosine phosphorylation of PHB in these pathways has not yet been explored.

3.8 Tyrosine phosphorylation of PHB in FSH signalling

Ovarian granulosa cells (GCs) play a vital role in the growth and development of ovarian follicles [13]. PHB is widely expressed in the ovary and its expression is dependent on the age of an organism and the stage of follicular development [13]. In a recent study, it has been shown that

follicle stimulating hormone (FSH) upregulates the expression of PHB in rat primary GCs [13]. In addition, PHB was phosphorylated at Tyr-249, Thr-258 and Tyr-259 during GC differentiation [13]. The observed level of phosphorylation at all three residues was found to be low in the absence of FSH stimulation, while enhanced phosphorylation was observed in the presence of FSH [13]. Furthermore, phosphorylation of PHB was inhibited by the use of PD98059, an inhibitor of the ERK pathway [13]. These experimental results collectively point out that PHB is a substrate of MEK and p38 MAPK during GC differentiation. It is interesting to note that Tyr-249 of PHB is located within the RAF1 binding domain, which spans amino acids 243-275 in PHB. Reports have demonstrated that PHB is indispensable for the activation of the RAF1/MEK/ERK pathway by Ras [29]. Low levels of RAF1 Ser-338 phosphorylation and high levels of Ser-259 phosphorylation are observed under low PHB condition [29]. Membrane targeting and activation of RAF1 by Ras require PHB *in vivo* [29]. In the absence of PHB, RAF1 kinase fails to interact with RAF1 upon activation by the EGF stimulation [29]. These results indicate that there might be a hierarchical relationship between PHB and the MEK/ERK pathway, and tyrosine phosphorylation of PHB might have an important role in regulating the Ras-mediated RAF1/MEK/ERK pathway. Cell fractionation in GCs revealed that only mitochondrial PHB became phosphorylated [13], which would imply that phosphorylation of PHB may occur in different cellular compartments and/or have a role in the intracellular trafficking of PHB. Taken together, these results provide new evidence that tyrosine phosphorylation of PHB plays an important role in regulating the FSH signalling pathway in ovarian GCs.

3.9 Tyr-114 phosphorylation of PHB in iron binding

Mitochondria play a central role in iron homeostasis. They are unique sites for heme synthesis and for iron-sulphur cluster biosynthesis [60]. Cellular proteins that are involved in iron homeostasis are often upregulated during oxidative stress. PHB has been reported to bind iron and is upregulated during oxidative stress. Specifically, Tyr-114 in PHB is an important residue for iron binding [61]. There are numerous reports supporting a protective role of PHB against oxidative stress and mitochondrial dysfunction [10,62,63]. It is possible that its protective effect is facilitated by the iron-binding function. Looking at Tyr-114 in PHB, it appears to perform functions unique to each cellular compartment. For example, plasma membrane-associated PHB is involved in cell signalling, whereas mitochondrial PHB may aid in iron binding. A number of tyrosine kinases, including EGF receptor, fibroblast growth factor receptors, and ErB2, can translocate to the mitochondria and phosphorylate PHB; the mitochondrial translocation of Src family kinases has been reported [64,65]. It is plausible that the phosphorylation of Tyr-114 in PHB modulates its iron-binding property and other mitochondrial attributes such as chaperone/scaffolding [Figure 3.4]. Moreover, our laboratory has recently shown that PHB has an important role in immune functions at the organismal level [66]. However, the underlying mechanism involved remains to be determined.

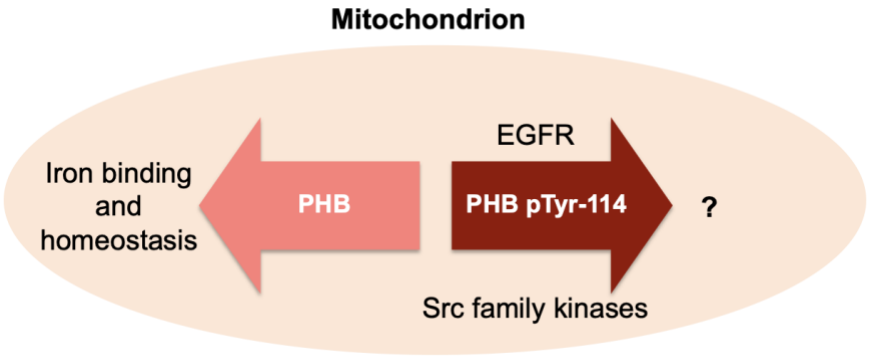

Figure 3.4 Does tyrosine phosphorylation of PHB (e.g., Tyr-114) play a role in iron binding or other potential mitochondrial attributes of PHB? A number of tyrosine kinases including epithelial growth factor receptor (EGFR) and Src family kinases that phosphorylate PHB are known to localize to the mitochondria. p: phosphorylated; Tyr: Tyrosine.

3.10 Mito-Ob and mMito-Ob mice: Transgenic mouse models of PHB

PHB has an important role in adipogenesis [9]. Our laboratory has shown that overexpression of PHB in 3T3-L1 pre-adipocytes induces adipocyte differentiation [9], whereas silencing of PHB in 3T3-L1 cells inhibits adipocyte differentiation [67]. MicroRNA-27, which targets PHB, impairs adipocyte differentiation and mitochondrial function when it is expressed in adipose-derived stem cells [68]. These results provide convincing evidence that PHB plays an important role in adipogenesis. To find the role of PHB in adipose tissue biology at the systemic level, a transgenic mouse named Mito-Ob overexpressing PHB in adipocytes was developed. As expected, Mito-Ob mice developed obesity independent of diet [69]. Interestingly, female Mito-Ob mice had normal glucose homeostasis and insulin sensitivity, whereas male Mito-Ob mice developed impaired glucose homeostasis, hyperinsulinemia and insulin resistance [69]. These results provide evidence that PHB plays a role in regulating adipose tissue homeostasis and facilitates sex-dimorphic functions.

As mentioned, PHB is phosphorylated at Tyr-114 in response to insulin, and this modification aids in the recruitment of Shp1, which is a negative regulator of insulin signalling [21]. Reports in the literature have also suggested that tyrosine phosphorylation of PHB has an important role in immune signalling, maturation and survival of T cells and in thymic growth [23,70]. More recently, PHB has been identified as an adapter protein in antigen receptor signalling in mast cells [51]. There, it facilitates protein-protein interaction and cytokine release in a Tyr-114 phosphorylation-dependent manner. These results emphasize that phosphorylation of PHB at Tyr-114 is crucial for controlling cell signalling-mediated events. To explore the physiological relevance of phosphorylation of PHB in immune functions at the systemic level, our laboratory developed a mutant mouse model (mMito-Ob) overexpressing Y114F-PHB from the *Fabp4* gene

promotor [28]. The *Fabp4* gene is primarily expressed in adipocytes but also selectively in macrophages and dendritic cells [66]. Similar to Mito-Ob mice, mMito-Ob mice developed obesity independent of diet in a sex-neutral manner, but only male mice developed glucose intolerance, hyperinsulinemia and insulin resistance [28]. These experimental results suggest that Tyr-114 mutant mice retained adipogenic functions, and tyrosine phosphorylation of PHB in the mitochondria may not play adipogenic functions. While both male Mito-Ob and mMito-Ob mice exhibited similar metabolic features such as obesity, insulin resistance, and adipose tissue inflammation, a fraction of the male Mito-Ob mice developed non-alcoholic steatohepatitis (NASH) and hepatocellular carcinoma (HCC) by 12 months of age [71]; whereas, some male mMito-Ob mice developed lymph node tumours by 6 months of age [28]. Development of histiocytosis with massive lymphadenopathy in mMito-Ob mice demonstrated that Tyr-114 phosphorylation of PHB had anti-proliferative effects on immune cells such as macrophages and dendritic cells [28], and consequently on B and T cells. This finding may, in part, be explained by the inhibitory effect of PHB Tyr-114 phosphorylation on PI3K/Akt signalling [21,22]. Interestingly, ovariectomy in female mMito-Ob mice resulted in glucose intolerance, hyperinsulinemia, insulin resistance and subsequent lymph node tumours similar to their male counterparts [28]. However, these events were not observed in ovariectomized Mito-Ob mice [28]. Collectively, results suggest that PHB and Tyr-114 phosphorylation in PHB act differently in the presence of estrogen [72]. It would be interesting to determine if the tyrosine phosphorylation of PHB has a role in estrogen-mediated cell signalling.

Although the mechanisms involved in PHB- and mutant PHB-induced metabolic and immune phenotypes in Mito-Ob and mMito-Ob mice remain to be elucidated, the interaction of PHB and OGT and the subsequent *O*-GlcNAc modification of PHB, insulin and immune signalling intermediates may play a role. *O*-GlcNAc modification targets a number of insulin signalling

intermediates and has been shown to promote insulin resistance [73–75]. Emerging evidence suggests that *O*-GlcNAc modification of immune signalling intermediates regulates their activity during cellular activation and differentiation [76]. For instance, *O*-GlcNAc modification of the NFκB subunit, c-Rel, is required for its DNA binding ability and induction of cytokine gene expression in activated T cells [77]. The inhibition of *O*-GlcNAc modification in this context is sufficient to repress *Il2* and *Ifng* expression following T cell receptor stimulation. *O*-GlcNAc modification of STAT3 exerts an inhibitory effect on STAT3 phosphorylation, which results in a loss of IL10 production in macrophages [78], thus demonstrating how *O*-GlcNAc modifications can control important transcriptional programs in immune cells. Of note, PHB has been shown to modulate NFκB and STAT3 signalling with a focus on phosphorylation-mediated events [79]. The potential role of *O*-GlcNAc modification and its crosstalk with phosphorylation has not yet been investigated.

3.11 Adult-onset type 1 diabetes in the male mMito-Ob mouse model

Obesity and its associated abnormalities appear to be an important factor in the development of adult-onset type 1 diabetes mellitus (T1D). However, the mechanisms behind the association between obesity and adult-onset T1D are not clearly understood. One surprising finding from Mito-Ob and mMito-Ob mouse models is that the male mMito-Ob mice developed autoimmune insulitis on a high-fat diet but male Mito-Ob mice did not [80]. Histological analysis of pancreatic islets from mMito-Ob mice revealed a progressive increase in infiltrating immune cells to the pancreatic islets. At an early stage, immune cells were present around the pancreatic islets of male mMito-Ob mice, pointing to peri-insulitis [80]. At the advanced stage, immune cells migrated into the pancreatic islets, indicating features of intra-insulitis [80]. Immunohistochemical studies

identified immune cells present around the islets to be CD8 and F4/80 positive, which are indicators of cytotoxic T cells and macrophages, respectively [80]. However, pancreatic islets from the female mMito-Ob mice fed a high-fat diet and male mMito-Ob mice fed a low-fat diet were negative for CD8 and F4/80 [80]. These experimental findings indicate a complex role of PHB in facilitating the immune and sex-specific manifestations of diseases, and mutant PHB lacking the Tyr-114 phosphorylation site may increase the susceptibility of adult-onset T1D. More specifically, a loss of PHB phosphorylation at Tyr-114 appears to activate diabetogenic factors in response to environmental triggers, such as a high-fat diet. Thus, Mito-Ob and mMito-Ob mice have created unique opportunities to gain insights into the role of obesity-related abnormalities in the adult-onset T1D and for defining the role of PHB in antigen presenting cells such as dendritic cells and macrophages. Dendritic cells and macrophages are important cells in the immune system involved in antigen presentation and processing [54,55]. These cells act as mediators between the adaptive and innate immune systems. In our transgenic models, both male and female Mito-Ob and mMito-Ob mice overexpress PHB and mutant PHB, respectively, in dendritic cells and macrophages, but only male mice develop immune dysregulation suggesting a sexually dimorphic role of PHB in these cell types [66,72]. These results also suggest an important role of PHB phosphorylation at Tyr-114 in regulating the function of dendritic cells and macrophages. It is possible that mutant PHB can affect macrophage and dendritic cell function in a sexually dimorphic manner or that mutant PHB-overexpressing macrophages and dendritic cells respond differently in male and female organisms. In summary, these findings provide a proof-of-concept that that obesity-related abnormalities promote the development of adult-onset T1D by coupling environmental factors with genetic susceptibility. It is possible that similar factors are involved in the development of adult-onset T1D

in humans. It is anticipated that the Mito-Ob and mMito-Ob mice will prove to be valuable tools for mechanistic studies uncovering the role of PHB in cell signalling pathways.

3.12 Conclusions and future perspectives

PHB has pleiotropic functions in cell proliferation, growth, signalling and death. Cellular events require proper signalling mechanisms to function effectively. This review emphasizes several recent developments in the study of PHB associated with cell signalling and highlights the role of PHB tyrosine phosphorylation in mediating these cell signalling processes [Figure 3.3]. Defects in the tyrosine phosphorylation of PHB can lead to the development of various diseases, such as diabetes mellitus and different types of cancer [28,70,71,80]. In this review, we have highlighted the regulatory role of tyrosine phosphorylation of PHB in insulin signalling, FSH signalling, immune cell signalling, and potentially in iron binding in mitochondria. The role of tyrosine phosphorylation of PHB in signalling mechanisms is complex. Several studies have shown that upon stimulation with various stimuli, PHB translocates to the plasma membrane and becomes phosphorylated [18,21,22], and further traffics between the plasma membrane, mitochondria and the nucleus [10,38,39]. It is possible that the phosphorylation of PHB plays a role in its intracellular trafficking. It is crucial to unravel the cellular compartment-specific functions of PHB, and the relationship between PHB present on the plasma membrane, mitochondria and the nucleus. The two novel transgenic mouse models our laboratory developed have revealed the hidden roles of PHB and its tyrosine phosphorylation in adipocyte and immune crosstalk. A fuller understanding of the roles and regulation of PHB, especially the phosphorylation of its different tyrosine residues may lead to the discovery of new therapeutic targets including signal transduction-targeting therapy for metabolic- and immune-related diseases.

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CHAPTER 4: PROHIBITIN: A NEW PLAYER IN IMMUNOMETABOLISM AND IN LINKING OBESITY AND INFLAMMATION WITH CANCER

Preface

Immunometabolism is a relatively new concept in the field of physiology. The ability of PHB to integrate immune activation with metabolic response suggests that it is a potential key player in the metabolic regulation of immune function. This chapter connects the field of immunometabolism and the role of sex steroids on sex dimorphism to a novel player, PHB, in integrating cell signalling with cell metabolism. Based on discoveries from Mito-Ob and mMito-Ob mice, we propose that PHB may serve as a unique target for sex-specific manipulation of immunometabolism. PHB is an established player in signal transduction and response to a variety of immune cells, including B cells, T cells and mast cells. Here, we performed a literature review into the current knowledge about PHB in immunomodulatory effects and the potential therapeutic outlook in inflammation and cancers. Subsequently, an understanding of PHB in linking obesity and inflammation with cancer is necessary to better inform the observed phenotypes in Mito-Ob and mMito-Ob mice.

Contributions of Co-Authors

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4.1 Abstract

Immunometabolism, which has important implications in cancer biology, has emerged as a major regulator of different immune cell types. Various factors that integrate metabolic switches within immune cells with signal-directed programs that promote or inhibit their functions remain largely unidentified. Furthermore, sex differences are known to exist in immune functions and cancer incidences in the body, and sex steroid hormones are an integral component of these differences. However, factors that mediate such differences, and the potential link between the two fundamental aspects of immune cell biology that contribute to sex differences in health and disease remain unexplored. New evidence derived from novel tissue-specific transgenic mouse models of prohibitin (PHB) has revealed its crucial role in sex differences in adipocyte and macrophage functions and a potential role in endocrine-immune crosstalk. This review provides a point of view on the emerging role of PHB in immune functions with a special focus on immunometabolism and the immunomodulatory effects of sex steroids. We propose that PHB plays a crucial role in integrating cell signalling events with metabolic switches and may serve as a potential target for cancer immunotherapeutics.

4.2 Introduction

Immunometabolism that incorporates both the role of immune cells in metabolic homeostasis and the impact of key metabolic pathways on immune cells has emerged as a major regulator of macrophages and dendritic cells, which are critical regulators of innate and adaptive immunity [1,2]. Immune cells that participate in inflammation and immune checkpoints exhibit a shift towards glycolysis, whereas anti-inflammatory cells display high levels of oxidative metabolism [1,3]. However, various factors and mechanisms involved in integrating cell signalling events with the shift in cell metabolism in response to diverse cues remain largely unidentified. Recent success in cancer immunotherapy has generated a greater interest in better defining the molecular aspects of macrophage and dendritic cell biology because of their crucial roles in immune checkpoints and cancer surveillance. The macrophages are responsible for sensing, integrating, and responding appropriately to myriad stimuli in their tissue milieu. Despite the protean nature of these stimuli, macrophage activity is channelled through two distinct response patterns, designated as M1-activated and M2-activated macrophages [4]. The M1-activated macrophages secrete large amounts of pro-inflammatory cytokines and express high levels of co-stimulatory molecules important in T cell activation [4]. Conversely, M2-activated macrophages have an anti-inflammatory phenotype and express numerous pattern recognition receptors [5,6]. The M1 phenotype is implicated in numerous inflammatory and metabolic conditions including different cancers; whereas, alternative activation is associated with wound healing, tissue remodelling, metabolic homeostasis, and atopic diseases [4,6]. Emerging evidence suggests that metabolic reprogramming is integral to the responses of macrophages and dendritic cells to critical changes in the environment [7,8]. Recent works have pointed out that key metabolic regulatory events in immune cells can be initiated not only by nutrient and oxygen conditions, but also by

programming events downstream of activated pattern recognition receptors, cytokine receptors, and antigen receptors [1–4]. The analyses of metabolic reprogramming in macrophages and dendritic cells have provided new insights into how these cells perform their functions such as cytokine production and antigen presentations related to host defence and cancer surveillance [7,8]. However, various factors that integrate these metabolic switches remain unknown to us. Furthermore, sex differences exist in immune functions and cancer incidences in the body, and sex steroid hormones are integral components of these differences [9–11]. Estrogen is known to have crucial immunomodulatory effects. However, factors that mediate the effects of sex steroids, especially the immunomodulatory role of estrogen, have not been well explored. It is anticipated that a better understanding of the relationship between these two fundamental aspects of immune cell functions may have major implications in improving cancer immunotherapy as well as in reducing their cytotoxic effects. Such information may also provide insights into the sex differences in incidences and mortality rates of different cancer types and in predicting the effectiveness of immunotherapy under conditions with disrupted immunity, such as obesity. In this mini-review, we will focus primarily on the emerging roles of PHB in adipocyte- and immune cell-specific functions and its potential relevance in cancer biology, as other attributes of PHB related to cancer biology have been reviewed recently [12–14]. We will describe this in light of new evidence from adipocyte- and immune cell-specific transgenic models of PHB. We will also discuss other attributes of PHB that make it a unique target for sex-specific manipulation of immunometabolism, which may have implications for tumour surveillance and cancer immunotherapy in a context-dependent manner.

4.3 PHB: A potential link between immunometabolism and sex differences in adipose and immune functions, and their relevance in cancer biology

4.3.1 PHB as an anti-proliferative and tumour suppressor gene

PHB was discovered in a quest for anti-proliferative genes, hence the name 'prohibitin' [15]. As a result, initial work on PHB was primarily focused on identifying mechanisms involved in its anti-proliferative function and its potential involvement in cancer development [13]. Such studies led to the discovery of p53 and Rb, two well-known tumour suppressors [16,17], as PHB's interacting partners. PHB was shown to increase the transcriptional activity of p53 [16], whereas, in the case of Rb, PHB was shown to repress the activity of the E2F transcription factor [17]. In this context, Koushyar *et al*. have recently demonstrated that the PHB's repressive interaction with E2F is inhibited by androgen signalling in prostate cancer cells (possibly in a phosphorylation-dependent manner) suggesting a potential mechanism of androgen receptor-mediated cancer cell proliferation [18]. In addition to the role of PHB as a transcriptional co-regulator in relation to its anti-proliferative activity, subsequent studies revealed that PHB is a pleiotropic protein with roles in many fundamental cellular processes, as well as cell type-specific functions [13,19]. The pleiotropic attributes of PHB are mediated in a cell-compartment- and tissue-specific manner, which include plasma membrane-associated cell signalling, mitochondrial chaperone, and transcriptional co-regulator in the nucleus [13,19]. These findings suggested new possibilities for the potential role of PHB in cancer biology in a context-dependent manner.

4.3.2 PHB in adipocyte biology and its potential link with cancer

The prevalence of obesity has risen steadily in the past decades. It was estimated that 15-20% of cancer incidence and deaths in some developed countries are attributed to being overweight

and obese [20–22]. The underlying mechanisms linking obesity with different types of cancer are not well understood. Several mechanisms for obesity-related cancer development and progression have been proposed, including obesity-related low-grade inflammation, insulin resistance, hormone dysregulation, and hypoxia-induced angiogenesis [23]. The major contributors within the adipose tissue to obesity-related cancer development are adipocytes, macrophages and their multifaceted bidirectional crosstalk [23,24]. Adipocyte hypertrophy leads to dysregulation of their normal functions, increased infiltration from both the innate and adaptive immune systems, inflammatory secretions, and altered endocrine functions [24,25]. Such alterations significantly affect macrophage dynamics and lead to chronic inflammation within the adipose tissue, which in turn affects adipocyte functions and creates a microenvironment favourable for tumour development [23,24]. The adipose tissue is susceptible to expressing receptors for cytokines, chemokines and growth factors in response to pro-inflammatory stimulation [24,25]. Taken together, inflammation and altered immune response are important components of obesity and contribute greatly to the promotion of obesity-related complications, including cancer development. Since there is a correlation between obesity and cancer incidence around the world, understanding their association is of great significance. Studies that underpin mechanisms by which obesity leads to cancer development and progression are needed to improve cancer prevention and management [23]. A better understanding of the mechanistic link between obesity and cancer is expected to identify intervention targets and strategies to avoid the pro-tumorigenic effects of obesity [24]. Unfortunately, there is a lack of obesity-linked cancer models that develop obesity independent of diet or cancer independent of carcinogens. It is rather difficult to distinguish the main drivers from the confounders, which often coexist in current approaches to study obesity-linked cancer.

A number of studies have shown the important role of PHB in adipocyte biology and metabolic homeostasis. For instance, overexpression of PHB in preadipocytes facilitates adipogenesis, whereas its knockdown attenuates adipogenesis [26–29]. The role of PHB in adipogenesis appears to be mediated through its mitochondrial function [27,28]. Knockdown of PHB in *C. elegans* results in diminished intestinal fat content, whereas the opposite effect was observed under mutation of the *daf2* gene (the insulin / insulin-like growth factor equivalent in *C. elegans*) [30]. An interpretation of this finding that extends to rodents and humans implies that interfering with PHB function in adipose tissues may affect metabolic homeostasis differently depending on the status of insulin sensitivity in the body [13]. In this context, it is important to note that PHB has been reported to localize to lipid droplets in response to hormone stimulation of lipolysis [31]. Interestingly, lipid droplets have been identified as highly dynamic organelles that participate in intracellular signalling and metabolic control [32]. In addition, PHB has been found to attenuate insulin-stimulated fatty acid oxidation in adipose tissue [33]. Of note, an analysis of the *Phb* promoter sequence has revealed the presence of putative insulin-response elements in the *Phb* gene [26]. Collectively, these findings point toward a strong relationship between PHB and insulin in the regulation of adipose tissue homeostasis and conceivably their dysregulation in metabolic impairment and cancer development. PHB is shown to be regulated by leptin in adipocytes [26]; overexpression of PHB in adipocytes alters the secretion of a number of adipokines in a sex-specific manner [27], including those that have been implicated in obesity-linked cancer development, such as leptin and resistin [13,19]. These findings indicate that impairment of PHB function in adipocytes leads to hormone dysregulation, which may facilitate cancer development and warrants further investigation. Obesity-linked insulin insensitivity and hyperinsulinemia together with associated low-grade chronic inflammation play a critical role in subsequent cancer development

[28,34]. In this context, it is important to note that Meijer *et al*. have reported that human primary adipocytes secrete pro-inflammatory mediators, prime inflammation independent of macrophages, and contribute to the conversion of regulatory macrophages to the proinflammatory form [25,35]. It appears that the primary event in the sequence leading to chronic inflammation in adipose tissue may be the metabolic dysfunction of adipocytes, which is then exacerbated by activated macrophages in the adipose tissue. It would be interesting to know whether alteration in PHB level or function induces similar changes in adipocytes.

4.3.3 PHB in immune function and inflammation

PHB was first identified in association with the IgM receptor in murine B cells [36] and subsequently in thymic involution during pregnancy in mice [37]. Although the precise role of PHB in IgM receptor signalling remained to be determined, a number of studies have reported an array of functions for PHB in different immune cell types, which include a role as an adaptor molecule in B cell receptor signalling [38], antigen-stimulated signalling in mast cells [39], and as an important protein for mitochondrial integrity and maturation of T cells [40]. PHB is also discussed in the context of mitochondrial stabilization and cell survival in hematologic malignancies [41]. Immune cell-specific functions of PHB including its role in thymic involution appear to require its phosphorylation at different residues [37–42]. In inflammatory diseases, intestinal epithelial cell-specific PHB overexpression from the *Vil 1* gene promoter has been shown to attenuate colonic inflammation in experimental models of colitis [43], whereas its deficiency in hepatocytes promotes inflammation and increases sensitivity to liver injury [44]. These results suggest a role of PHB in the innate immune response, as it was not directly manipulated in immune cells. Of note, an analysis of the *PHB* promoter sequence has revealed the presence of a putative IL-6 response element [45].

Moreover, PHB has a modulatory role in STAT3 signalling [46], which is known to play a role in mitochondrial biology and cell signalling related to macrophage polarization [47,48]. Thus, a multifaceted relationship of PHB with molecular events important to the functional plasticity of immune cells implies that PHB plays a role in signal integration that guide cell physiology and functional output.

Additional evidence revealing a role of PHB in the immune system came from studies that identified PHB as a host target protein for a number of pathogens [49–52]. For instance, the virulence polysaccharide Vi of *Salmonella typhi* interacts with the membrane PHB complex and inhibits T cell receptor signalling [50]. It is suspected that targeting PHB provides a survival advantage for the pathogens, which may include metabolic benefit, immune evasion, or a combination of both [13,19]. The impairment of PHB function induces oxidative damage, which may be involved in the disease process in viral hepatitis [53] inflammatory bowel disease [43], as well as impaired insulin secretion from pancreatic beta cells [54]. More recently, Tortelli *et al*. have reported that the accumulation of PHB in melanoma cells is a common cellular response to different stress stimuli and protects them from chemotherapy-induced cell death [55]. However, the biological and physiological relevance of PHB in metabolic and immune functions at the organismic level have only recently been uncovered. Of note, alterations in adipose-derived factors have been reported in inflammation and type 2 diabetes, and dysfunction in metabolic energetics is considered the main cause of type 2 diabetes [56]. For instance, the expression of caveolin 1 in human adipose tissue is upregulated in obesity, obesity-associated type 2 diabetes and inflammation [57]. Interestingly, circulating lipopolysaccharide binding protein has been identified as a marker of obesity-related insulin resistance [58]. Furthermore, gene expressions of major lipogenic enzymes are downregulated in the visceral adipose tissue of obese subjects [59]. Collectively, these findings have provided important

clues to the relationship between obesity, insulin resistance, type 2 diabetes, and cancer. As type 2 diabetes and cancer are frequent comorbidities of obese patients, obesity and insulin resistance are important factors that may promote cancer development [60].

4.3.4 PHB has a role in sex differences in adipose and immune functions

In an effort to better define the molecular mechanisms involved in the role and regulation of PHB, our laboratory has identified Tyr-114 residue as an important phosphorylation site in PHB [61]. The phosphorylation of PHB at Tyr-114 residue creates a docking site for interacting partners that contain the SH2 domain, such as Shp1 phosphatase and the Src family of cytoplasmic tyrosine kinases (Lyn, Syk and Fyn) [39,42]. These cell signalling molecules are known to play important roles in various immune cell types including macrophages and dendritic cells [48]. At the systemic level, findings from the Mito-Ob and mMito-Ob mice revealed that Mito-Ob and mMito-Ob mice differ in their immune phenotypes, where only male mMito-Ob mice developed lymph node tumours and splenomegaly, showing an increased proliferation of macrophages and dendritic cells in the lymph nodes, as well lymphocytes in the spleens [62]. While the male Mito-Ob mice developed obesity-linked non-alcoholic fatty liver disease and hepatocellular carcinoma (HCC) with aging [34], the female Mito-Ob and mMito-Ob mice remain protected from obesity-related metabolic and immune dysregulation [28,34,62]. These findings point to a protective role of estrogen against obesity-related metabolic and immune dysregulation in female Mito-Ob and mMito-Ob mice. These findings also indicate a relationship between PHB and estrogen actions in adipose and immune functions. Specifically, the phosphorylation of PHB at Tyr-114 plays a crucial role in macrophage and dendritic cell biology and in the regulation of lymphocyte numbers and functions [62]. To support this, ovariectomy in female mMito-Ob mice resulted in the development of lymph node tumours

similar to those of male mMito-Ob mice [62]. It is likely that estrogen modulates PHB function in macrophages and dendritic cells since PHB and its homologous protein PHB2 are known to interact with sex steroids [13]. Both PHB and PHB2 have been reported to function as a repressor of sex steroid action and have also been identified as target genes of sex steroids [66–69]. This relationship has mainly been reported in reproductive tissues [66–69]. However, the relevance of this relationship between PHB and sex steroids in immune cells remains unexplored. New findings from Mito-Ob mice and mMito-Ob mice suggest that the relationship between PHB and sex steroids is multifaceted [13] and may function as part of an autoregulatory loop to maintain tissue homeostasis.

4.3.5 PHB as a potential metabolic switch related to immunometabolism

The AMPK and Akt signalling pathways play crucial roles in macrophages, including modulations between the two different functional states of macrophages [70,71]. In this context, it is important to note that PHB participates in Akt signalling in a phosphorylation-dependent manner [61,72]. The phosphorylation of PHB at Tyr-114 negatively regulates Akt signalling whereas phosphorylation at Thr-258 has the opposite effect [61,72]. These two phosphorylation sites within PHB also negatively regulate each other [73,74]. New evidence suggests that PHB interacts with AMPK and plays a role in AMPK signalling [75,76]. Furthermore, PHB facilitates STAT3 and ERK signalling, which mediate the effects of IL-10 and M-CSF in macrophages, respectively [48,74]. It is conceivable that different immune phenotypes observed in Mito-Ob and mMito-Ob mice may be due to the effects of PHB on cell signalling cascades and integration in macrophages [Figure 4.1]. Possible mechanisms may involve direct interaction between membrane PHB and cell signalling intermediates or indirect interaction through other phosphorylation-dependent partners. A

potential role of mitochondrial PHB may not be ruled out because PI3K/Akt, MEK/ERK, and STAT signalling are known to regulate mitochondrial functions [1,77]. Thus, the membrane and mitochondrial functions of PHB may be interlinked. For example, mitochondria and the plasma membrane are reported to coalesce at the immunological synapse during T cell activation, likely to exchange membrane proteins [78]. Another interesting finding from mMito-Ob mice is the development of autoimmune insulitis on a high-fat diet in a male sex-specific manner [79]. Notably, this was not found in Mito-Ob mice, which shared the metabolic features of mMito-Ob mice including male sex-specific obesity-related metabolic dysregulation [62,79]. The dramatic alterations in immune phenotypes in response to environmental stresses in mMito-Ob mice further strengthen the hypothesis that PHB and its phosphorylation at Tyr-114 have crucial roles in the functional plasticity of macrophage and dendritic cell functions including self-tolerance and tumour surveillance. Consistent with our *in vivo* findings, recently we have found that manipulation of PHB levels or its functions related to the Tyr-114 phosphorylation site in murine macrophages (RAW 264.7 cells) differently alters macrophage polarization, mitochondrial respiration, and its response to sex steroids (unpublished data). Furthermore, we found that PHB and Y114F-PHB differently affect the Akt, JAK1/3, ERK1/2 and STAT3/5/6 signalling in macrophages in the presence and absence of sex steroids, indicating a role of PHB in integrating cell signalling events with cell metabolism, and in the immunomodulatory effects of sex steroids (unpublished data). Taken together, these findings suggest that PHB may play a crucial role in the functional plasticity of macrophages and the immunomodulatory role of sex steroids [Figure 4.1]. It would be interesting to know whether macrophage- and dendritic cell-specific functions of PHB are mediated through metabolic switches involving its mitochondrial functions or membrane

signalling functions, or a combination of both. In addition, it is important to find out whether this attribute of PHB contributes to its anti- or pro-tumorigenic effects in the tumour microenvironment.

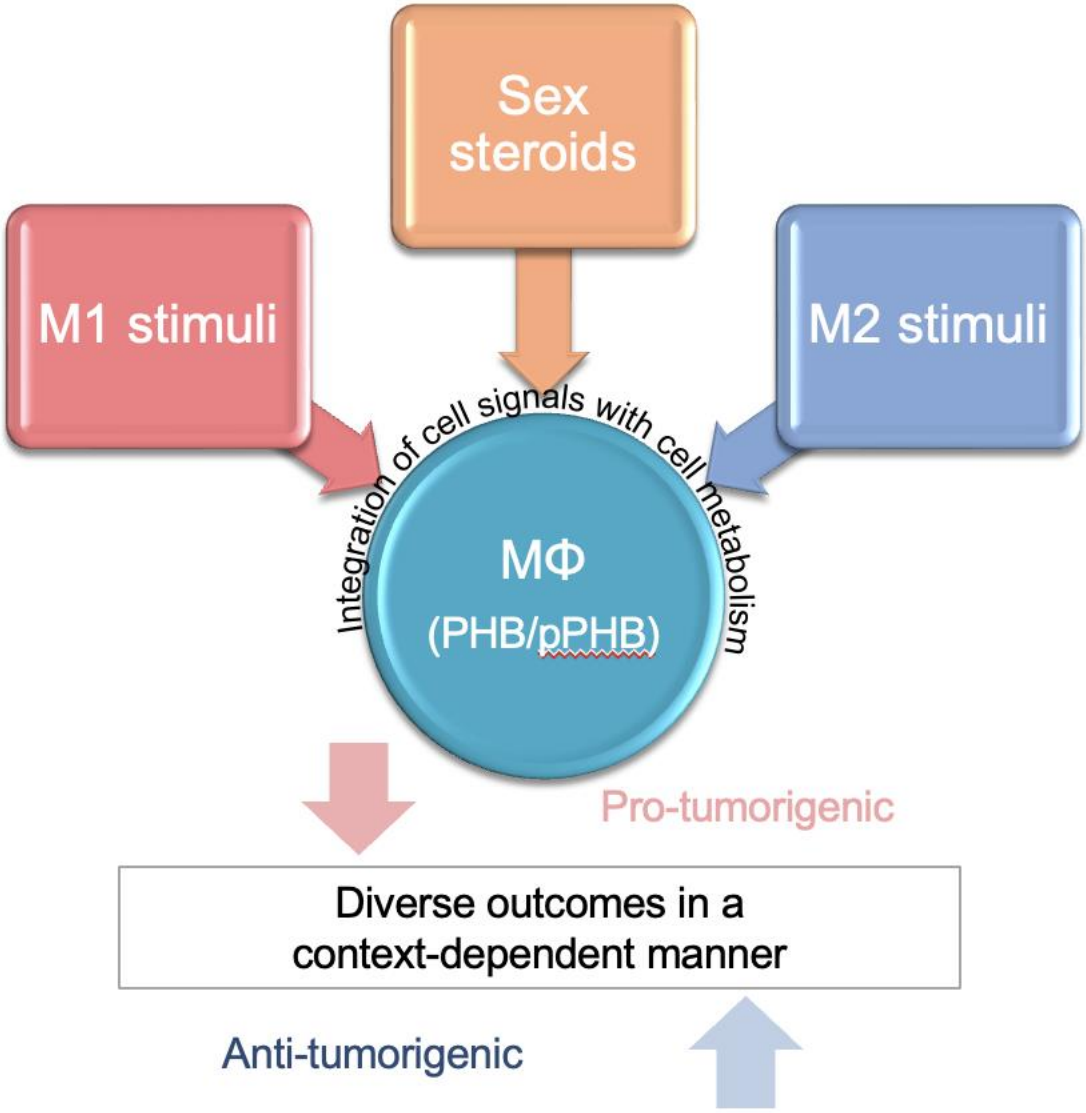

Figure 4.1. A schematic diagram of the emerging role of prohibitin (PHB) and its phosphorylation in integrating the immunomodulatory effects of sex steroids with immunometabolism in macrophages and their potential relationship with anti- and pro-tumorigenic functions. MΦ: Macrophages; M1: Proinflammatory macrophages; M2: Anti-inflammatory macrophages; p: Phosphorylated.

4.4 Sex steroid hormones in immune functions

Sex steroid hormones exert potent effects on immune cell subsets [80]. For instance, the global expression profile of peripheral blood mononuclear cells from both young and elderly people has revealed age- and sex-dependent changes in immune cell transcriptomes [81]. Accumulating evidence from animal models and human studies supports a role for sex-based differences in various aspects of immune cell functions [Table 4.1] [82–94], including susceptibility and resistance to infectious diseases, autoimmune diseases, and different types of cancers [95,96]. While such differences may be due to a number of factors, sex steroid hormones appear to play a major role in it. This is because changes in sex steroid levels and immune-related alterations occur in parallel to various stages of life, such as pre-puberty, puberty, pregnancy, and aging [97]. Notably, sex differences in immune responsiveness are most accentuated post-puberty [98,99] and substantially modulated during pregnancy, suggesting that sex hormones play a crucial role in it. Maternal immunomodulation is important for the tolerance of a semi-allogeneic fetus and the success of pregnancy depends mostly on synchronized endocrine-immune crosstalk [97]. Hormones are important in maintaining a suitable environment and sufficient nutrition for the developing fetus. This is achieved in synchronization with a variety of endocrine stimulations. Estrogen, progesterone, and human chorionic gonadotropin (hCG) all take part in immune modulation during pregnancy [97], and dysregulation of their actions is known to play roles in the development and progression of a number of different types of cancer in adults [100,101]. Sex-specific tumour or autoimmune insulitis development in Mito-Ob and mMito-Ob mice in a context-dependent manner [34,62,79] suggest a relationship between sex steroids and PHB in the regulation of immune function related to tumour surveillance and self-tolerance. Thus, Mito-Ob and mMito-Ob mice have

revealed a potentially novel mechanism related to the tumorigenic function of PHB that warrant further investigation.

Table 4.1. Sex differences in different types of immune cells.

Type of Immune cells	Characteristics	Sex Differences [References)
B cells	B cell numbers	Higher in females [84]
Dendritic cells	Type 1 interferon activity	Increased in females upon stimulation with TLR7 [82], [83]
	Activity of TLR7	Increased in females [84]
Macrophages	Activation	Higher in females [86]
	Phagocytic capacity	Higher in females [87]
	Pro-inflammatory cytokine production	Stimulation with LPS results in higher cytokine production in males [88]
	TLR4 expression	Higher in males [89]
Neutrophils	Cell numbers	Higher in females [85]
	Phagocytic capacity	Higher in males [87]
	TLR expression	Higher in females [90]
NK cells	Cell numbers	Higher in males [85]
Plasma cells	Antibody production	Higher in females [85]
T cells	$CD4^+$ T cells count	Higher in females [91], [92]
	$CD8^+$ T cells count	Higher in males [91]
	CD4/CD8 Ratio	Higher in females [91], [92]
	Cytotoxic T cells	Increased cytotoxic activity in females when stimulated with PMA- ionomycin [93]
	Number of activated T cells	Higher in females [86]
	T regulatory cell count	More in males [94]

4.4.1 Sex steroids in macrophage functions

Using a transcriptomic approach Pepe *et al*. have reported a dynamic nature of the murine macrophage response to estradiol, and specific biological programs induced by the hormone, with cell proliferation, immune response, and wound healing being the most prominent functional categories [102]. In particular, the endogenous hormonal surge has been shown to support macrophage proliferation *in vivo*. These findings highlighted the direct effect that estrogen has on

macrophage expansion and phenotypic adaptation in homeostatic conditions and suggested a role of this interplay in inflammatory pathologies. Using human blood monocyte-derived macrophages, Campesi *et al.* have shown that macrophages from men and women express both estrogen receptors (ERα and ERβ), and ERα plays a significant role in LPS-induced inflammatory response [103]. In addition to the genomic action of estradiol, the role of G protein-coupled estrogen receptor (GPER) has also been demonstrated. Wei *et al*. have shown that GPER knockout in a diethylnitrosoamine (DEN)-induced mouse tumour model significantly accelerates liver tumorigenesis, accompanied by enhanced immune cell infiltration and production of inflammatory cytokine IL-6 [104]. Moreover, they found that treatment with selective GPER agonists decreases the expression of IL-6 in bone marrow-derived macrophages, which is abrogated upon deficiency of GPER [104]. These findings provided strong evidence of GPER's presence in the macrophage response. Sex steroid hormones are known to play a role in sex differences in hepatocellular carcinoma (HCC) development that is known to exist in both rodents and humans [34]. Moreover, a high level of IL-6 has been implicated in sex differences in HCC development [105]. As male Mito-Ob mice display higher IL-6 level and develop obesity-linked HCC, it would be interesting to determine whether the relationship between sex steroids and PHB have a role in sex differences in HCC development.

An important immunomodulatory effect of sex steroids is further supported by the fact that hormonal fluctuations accompanying the menstrual cycle, pregnancy, and menopause have an impact on immune responses and susceptibility to infectious diseases [80]. However, it remains unclear how hormonal changes during different reproductive stages influence the immune phenotypes, which then create alterations in immune response and susceptibility. The ways in

which sex-dependent-immune cell phenotypes are influenced by sex hormones is an emerging topic of study, and freshly accumulating data are providing new insights [80].

4.4.2 Sex steroids in dendritic cell functions

Dendritic cells are immunological sentinels that link innate immunity to adaptive immunity [106]. Dendritic cells have a central role in the activation of the adaptive immune response, and in the maintenance of tolerance. Female sex steroid hormones estradiol and progesterone play a key role in regulating immune responses in women, including dendritic cell development and functions [107–109]. Although the two hormones co-occur in the bodies of women throughout the reproductive years, their physiological concentrations and ratio vary substantially during the reproductive cycle as well as during pregnancy. Given their ability to regulate each other's actions, it is possible that they may exert a wide range of immunomodulatory roles during the reproductive cycle and stage [86]. Available information in the literature suggests that this is indeed the case. For instance, Xiu *et al*. have shown that estradiol enhances the differentiation of $CD11b^{+}CD11c^{+}$ dendritic cells from bone marrow precursor cells and promotes the expression of CD40 and MHC class-II, in a dose-dependent manner [110]. In contrast, progesterone inhibits dendritic cell differentiation, but only at high concentrations. In addition, a higher concentration of progesterone, at levels seen in pregnancy, reverses the effect of estradiol, regardless of the concentration of estradiol [110]. A parallel effect on dendritic cell function has also been reported such as pro-inflammatory cytokine production [110]. A dominating effect of higher physiological concentrations of progesterone provides insight into how dendritic cell functions could be modulated during pregnancy.

Furthermore, a similar effect of estradiol on interferon alpha (IFNα) production from plasmacytoid dendritic cells has been reported in response to Toll-like receptor activation in mice. Genetic deletion of the ERα gene in dendritic cell lineage abrogates the enhancing effect of

estradiol on IFNα in response to Toll-like receptor activation, indicating that estradiol directly targets dendritic cells *in vivo* [111]. A similar effect has also been reported in postmenopausal women in response to estradiol treatment indicating a conserved pathway of the estradiol effect on plasmacytoid dendritic cells, which may account for sex differences in autoimmune and infectious diseases, as well as different types of cancer. Collectively, these findings suggest that macrophages and dendritic cells may be a part of the immune cell subset that is highly sensitive to sex steroid hormones, and therefore, a target for future sex-based immunotherapies for different types of cancer.

4.4.3 Do sex differences in immune functions contribute to sex differences in cancer incidence?

The sex difference in cancer susceptibility is one of the most consistent findings in cancer epidemiology worldwide [112]. For instance, hematological malignancies are generally more common in males, and this generalization can be applied to most other cancers [113,114]. Furthermore, males have poorer overall survival and higher mortality rates in diseased states [116,117]. Similar sex differences exist in autoimmunity. Unlike cancers, autoimmune diseases are more commonly observed in females [80,86]. The pattern that autoimmune disorders are more common in females but cancer and infectious diseases in males suggest differences in immunity may be responsible for this dichotomy [13,19]. The sex difference in cancer incidence is comparable to ethnic and racial disparity in magnitude [112]. Yet, most studies fail to look for it. Sex difference in susceptibility to a disease is useful in formulating hypotheses for disease development and progression and in identifying subgroups at risk [115]. This difference may lie in the male and female immune systems since immune surveillance against infectious agents and cancer cells share similarities [112]. In this context, it would be interesting to investigate whether male and female Mito-Ob and mMito-Ob mice have differential susceptibility to pathogens and carcinogens.

Physiological differences between males and females may explain why there is a disparity in the incidence of certain cancers. For instance, a higher incidence of HCC in males may be attributed to higher IL-6 levels in males. IL-6 aids in the progression of HCC; estrogens inhibit IL-6 production, which lower the risk of HCC in females [105,118]. In this context, it is important to note that the main physiological differences between males and females are facilitated by sex hormones, but sexual dimorphism is present at many levels including gene expression, metabolomics, and proteomics [112]. The Mito-Ob mice display many features of HCC in humans, including higher IL-6 levels in males [34]; thus, they have created an opportunity to examine the molecular underpinning of sex differences in HCC incidence.

Both estrogens and androgens have been recognized as modulators of the immune response and determinants of sex differences in disease susceptibility [119,120]. Estrogen receptors are present in B and T cells, neutrophils, macrophages, NK cells and thymic stromal cells [80,86]. Membrane-bound forms of estrogen receptors have different functions and are present in lymphocytes and macrophages [121]. The effect of sex steroids on immune cell functions is generally achieved by their influences on cell signalling pathways, including NFκB and interferon regulatory factors [122]. In addition to sex steroids, the X chromosome may also contribute to sex differences in immune functions, because the X chromosome contains not only the largest number of immune-related genes in the whole human genome [123,124], but it also contains microRNAs with roles in the immune system [125]. Thus, the combined effect of X chromosome-associated immune-related genes and estrogens may contribute significantly to sex differences in cancer incidence and mortality.

New findings from the Mito-Ob and mMito-Ob mice suggest a crucial role of PHB in mediating the effects of sex steroids on adipose and immune functions, which warrants further investigations. It is possible that dysregulation of the intricate relationship between sex steroid

hormones and adipose-immune function may contribute to sex differences in cancer incidence. The appearance of metabolic dysregulation and lymph node tumour development in the gonadectomized female mMito-Ob transgenic mice, despite the reversal of obesity [62], suggests a crucial role of PHB in mediating the effects of sex steroids in adipose and immune functions at the systemic level. These novel transgenic mice have created opportunities to further define the relationship between sex steroids and adipose-immune functions, especially in the context of obesity, and their relative contribution to the development of obesity-linked cancer. It would be interesting to know whether sex differences in adipose and immune functions in humans have a role in sex differences in cancer incidence.

Immune surveillance has now been recognized as a major physiologic mechanism protecting our bodies from cancer development and progression [126,127]. Since sexual dimorphism in the immune response capacity is also well recognized, the difference in immune surveillance competence between males and females should contribute to the overall sex effect in cancer incidence. In cancer, susceptibility is generally higher in men although some cancers are more common in women. The same is true for autoimmunity, in which females have an overall higher susceptibility, but males are more susceptible to developing certain autoimmune diseases [80,86]. The endogenous causes of the sex differences observed in cancer and autoimmune diseases are largely unknown. Over the last decade, substantial progress has been made in the field of immunometabolism and cancer immunotherapies, and more progress should be expected during the next decade. It is anticipated that the Mito-Ob and mMito-Ob mice will prove valuable tools to advance our understanding of the relationship between immunometabolism and sex differences in immune functions, and their contributions in sex differences in cancer incidence and mortality worldwide.

4.5 Conclusions

The field of immunometabolism has grown substantially over the last decade, revealing the major roles that immune cells play in metabolic homeostasis at the organismic level and the role of key metabolic pathways in immune cell biology. As we gain a better understanding of how local and systemic metabolism are integrated at the cellular level and how immune cell functions are regulated, new therapeutic opportunities are likely to emerge for a wide range of diseases, including metabolic diseases, autoimmunity, and cancer. The discovery that PHB plays a crucial role in sex differences in adipose and immune functions and in integrating metabolic and immune functions in a sex-specific manner is an important step in this direction [28,34,62,79] [Figure 4.1]. Further studies dissecting the relationship between sex steroid hormones and PHB in metabolic and immune functions may provide untapped opportunities targeting immunometabolism in a sex-specific manner, as various cancer types that involve their dysregulation often display sex differences in susceptibility or resistance.

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CHAPTER 5: THE ROLE OF PROHIBITIN IN THE FUNCTIONAL PLASTICITY OF MACROPHAGES

Preface

The *Fabp4* gene promoter allowed the simultaneous manipulation of adipogenic and immune signalling functions in Mito-Ob and mMito-Ob mice. During my master's degree, I examined PHB function in adipocytes and discovered sex- and adipose depot-specific roles of PHB during adipocyte differentiation. The results partially explained the phenotypes observed in male and female Mito-Ob mice but didn't explore the immunopathology observed in mMito-Ob mice. Therefore, investigations are performed in macrophages to complete our understanding of the complex obese microenvironment that exists in Mito-Ob and mMito-Ob mice, which led to the differential outcomes we observed and described.

Contributions of Co-Authors

Yang Xin Zi Xu: Experimental design, experimental protocol, animal experiments, data collection, data analysis, figure preparation, manuscript preparation

Sudharsana Rao Ande: Experimental design, animal experiments, data collection, data analysis

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5.1 Abstract

Immunometabolism plays a crucial role in the activation and functional plasticity of immune cells, which in large determines a variety of health and disease states. Factors that integrate immunometabolism in immune cell signalling and functions are beginning to be identified. Previously, we have reported that two transgenic mouse models, Mito-Ob and mutant Mito-Ob (mMito-Ob), overexpressing a pleiotropic protein, prohibitin (PHB) or a mutant form of PHB (Tyr114Phe-PHB or mPHB), respectively, developed distinct immunometabolic phenotypes. Specifically, the immune phenotype appears to be driven by the monocytic cell lineage. Based on immunophenotyping of their splenocytes, we focused our attention on macrophages and hypothesized that PHB may play a role in regulating the two functionally polarized states, M1 and M2. Here, we report that macrophage polarization to the M1 and M2 phenotypes did not alter the PHB protein level, but overexpression of PHB in macrophages differentially affected cytokine production in the two polarized states. Furthermore, we found that mutation of the Tyr-114 phosphorylation site in PHB affects ERK and STAT6 signalling, arginase synthesis and activity, and mitochondrial respiration in macrophages indicating an important role of PHB in integrating cell signalling events with cell metabolism. In summary, we have discovered that PHB is a crucial regulator in the functional plasticity of macrophages. These initial studies expect to lay the foundation for future research into the relationship between cell signalling events pertaining to

immunometabolism in immune cell functions, which are integral components of immune-related health and disease.

5.2 Introduction

The immune system has been recognized as an active participant of the endocrine and metabolic system [1]. Among the many cell types in the immune system, macrophages have attracted interest due to their enormous plasticity in tissue homeostasis and the etiology of metabolic diseases and cancers. Macrophages can travel in the general and lymphatic circulations or take up residence and adapt to a specific tissue type. Tissue macrophages are known to maintain the "status quo" of a healthy body while monocytic macrophages rapidly extravasate and mobilize during a microbial invasion [2]. The plasticity of macrophages is heavily influenced by local and systemic signals, which alter their metabolic pathways for survival and functions [3]. Although considered a simplification, the M1/M2 dichotomy produces two well-characterized activated states of macrophages that are at two ends of a phenotypic continuum. The classically activated M1 macrophages typically stimulated by TNFα, IFNγ, and LPS amount to a highly aggressive phenotype against pathogens or during inflammatory stress, and secrete cytokines such as IL-1, TNFα, IL-6, IL-23, inducible nitric oxide synthase (iNOS) and chemokines [4]. The alternatively activated M2 macrophages typically stimulated by IL-4 or IL-13 are responsible for helminthic immunity, allergic response, and immunomodulation with increased production of anti-inflammatory molecules, such as IL-10, TGFβ, VEGF and others with the goal of tissue repair [4]. The two phenotypes utilize different metabolic pathways to generate energy and metabolites for survival and proliferation: the M1 macrophages shift towards glycolysis and enhanced pentose phosphate pathway, while the M2 macrophages favour fatty acid oxidation, oxidative

phosphorylation, and mitochondrial respiration [5,6]. These two polarized states of macrophages may interconvert under changing conditions but can be robustly defined by a set of unique mechanistic players involved in each pathway. Currently, the various factors that integrate metabolic switches within immune cells that promote or inhibit their functions are beginning to be discovered. A greater understanding of these factors involved in the functional plasticity of macrophages will enable us to harness the full potential of this cell type in normal body physiology and therapeutics against related diseases such as obesity, diabetes, and cancer.

Prohibitin (PHB or PHB1) is an evolutionarily conserved and pleiotropic protein that has cell compartment- and tissue-specific functions [7,8]. The best-characterized function of PHB is in the mitochondria as a scaffold protein, where it forms a multimeric complex with its homolog protein, prohibitin 2 (PHB2). PHB contains a nuclear export site and has been found in the nucleus as a transcription co-regulator [9]. On the plasma membrane, PHB undergoes post-translational modifications, most notably at tyrosine Tyr-114 and threonine Thr-258, as part of the PI3K/Akt and MEK-ERK signalling pathways, respectively [10,11]. Furthermore, PHB has been identified as a host target protein by a number of pathogens, indicating a putative role in molecular events involved in immune defence [12–14]. It is at the plasma membrane where PHB's immune-associated roles have been identified, mostly in support of the cell surface receptors [15]. The PHBs were independently discovered as B cell receptor-associated protein (BAP)-32 and -37 in relation to the IgM membrane isotype [16]. PHB/PHB2 expression was upregulated after CD40 priming in resting B cells. Specifically, the Syk tyrosine kinase, an intermediate in B cell antigen receptor signalling, interacts with PHB/PHB2 in a phosphorylation-dependent manner and leads to subsequent nuclear translocation of NFκB and increase in IgG1 production [17]. In T cells, the PHB/PHB2 complex increased in expression and localized with CD3 in leukemia T cells after activation by phorbol

myristate acetate and ionomycin, which suggests the involvement of PHBs in T-cell receptor signalling as well [18]. In mast cells, PHB was found primarily in the intracellular granules [19]. Upon stimulation by IgE, PHB was observed to translocate to the plasma membrane through palmitoylation at its only cysteine residue, Cys-69, within the PHB domain, followed by Lyn tyrosine kinase-mediated phosphorylation [19]. Further point mutations in PHB indicated that Tyr-114 and Tyr-259 sites were required for the recruitment of Syk tyrosine kinase and the degranulation of mast cell [19]. We have also identified a number of posttranslational modification sites in PHB that have important functions in regulating cell signalling [10,11,20]; one of them is Tyr-114 residue. Emerging evidence from the transgenic mouse models expressing PHB (Mito-Ob mice) or Y114F-PHB (mMito-Ob mice) from the fatty acid binding protein 4 (*Fabp4*) gene promoter suggest that the phosphorylation of PHB at the Tyr-114 site plays an important role in immune cell functions [8,21]. From these studies, male mouse models shared metabolic phenotypes but differed in immune phenotypes: the male mMito-Ob mice developed enlarged lymph nodes and splenomegaly, showing an increased proliferation of cells in the monocytic lineage, whereas the male Mito-Ob mice were spared. Moreover, Mito-Ob and mMito-Ob mice display differential cytokine levels (e.g., IL-2, IL-6, IL10, IFNγ and TNFα) and chemokine levels (e.g., SDF1, CCL21 and GCP2) in comparison with wild-type mice [7]. The *Fabp4* gene is known to be primarily expressed in adipocytes, as well as in mature macrophages and dendritic cells [22,23]. These findings prompted us to examine the role of PHB and the phosphorylation site at Tyr-114 in macrophages with the goal of better understanding its role in metabolic and immune functions. Our results pointed toward the potential role of PHB in integrating cell signalling with immunometabolism in the functional plasticity of macrophages.

5.3. Materials and methods

5.3.1 Mito-Ob and mMito-Ob mouse models

The development and phenotypic characterization of the Mito-Ob and mMito-Ob mice have been described previously [7,8,21]. Briefly, Mito-Ob and mMito-Ob transgenic mouse models are characterized by an increase in white adipose tissue mass due to PHB-induced upregulation of mitochondrial biogenesis. The transgenic gene construct was made by linking *Phb* cDNA to the regulatory region of the *Fabp4* gene promoter. Founder animals were identified by genotyping the tail genomic DNA using the following forward primer: 5′-GCAGCCCGGGGGATCCACTA-3′ and reverse primer: 5′-GCACACGCTCATCAAAGTCCTCTCCGATGCTG-3′. Founder animals were then mated with CD-1 female mice to establish PHB transgenic mouse lines as per standard procedures. Mito-Ob mice, mMito-Ob mice and their wild-type CD-1 counterparts were each caged in groups of 4, and allowed normal chow (LabDiet, St. Louis, MO) and water ad libitum. Changes in serum cytokine (e.g., IL-2, IL-6, IL-10, IL-12, IL-15, IL-18, IFNγ and TNFα) and chemokine (e.g., SDF1, CCL21, CXCL10, CXCL11, MCP2 and GCP2) profiles led to the current study. All experiments involving animals were carried out in accordance with the Animal Lab Requirements as per Animal Use Protocol approved by the Animal Care and Use Committee at the University of Manitoba and in accordance with the guidelines of the Canadian Council of Animal Care (CCAC).

5.3.2 Immunoprofiling

The spleen of wild-type (CD-1), Mito-Ob and mMito-Ob mice between 22 and 24 weeks old were harvested. Single-cell suspensions of splenocytes were made by mashing the splenic tissues and filtering resultant cells through a 70-µm cell strainer. The cells were stained with the

fluorescently labelled anti-F4/80-PE-Cy7 (BM8), anti-CD11c-Pacific blue (N418), anti-CD49b-FITC (DX5), anti-CD4-APC (RM4-5), anti-CD8-PE (53-6.7) and anti-B220/CD45R-PerCP-Cyanine5.5 (RA3-6B2) antibodies for 25min at 4°C in 20µl of staining buffer (2% fetal bovine serum and 0.01% $NaNO_3$ in PBS). Following the staining, the cells were then washed and analyzed on a FACS Canto-II flow cytometry analyzer 3 laser system (BD Biosciences). The cell populations were analyzed using FlowJo software (BD Biosciences).

5.3.3 Cell culture and treatment

RAW 264.7 cells (a murine model macrophage cell line) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and in Dulbecco's Modified Eagle Medium (ATCC) medium supplemented with 10% fetal bovine serum (Sigma) and 1% Penicillin/Streptomycin (Gibco) at 37°C with 5% CO_2 in a humidified cell culture incubator. After 24h of transfection, cells were stimulated with LPS (Sigma, 10 ng/ml), or recombinant mouse IL-4 (Sigma, 20ng/ml) for up to 24h [24,25]. 0.1% Bovine serum albumin (BSA)-stimulated cells were used as control. Serum-free cell culture medium was collected for cytokine analysis and nitrite production. Cell lysates were used for mRNA and protein analysis.

5.3.4 PHB overexpression and knockdown

RAW 264.7 cells were transfected at 70% confluency with pCMV6-XL5 vector containing human PHB or Y114F-PHB clone for overexpression[10] and scrambled shRNA or PHB shRNA (Origene, TG310467) for knockdown using X-tremeGENE HP DNA transfection reagent (Roche Applied Science, Laval, Canada) according to manufacturer's protocol. Overexpression and knockdown efficiencies were validated by detection of green fluorescent protein tagged-PHB and by immunoblotting.

5.3.5 Nitric oxide measurements

RAW 264.7 cells were seeded at a density of 5×10^5 cells in culture medium on a 12-well plate. After stimulation with LPS or IL-4 for 24h, the quantity of nitrites (NO_2^-) in cell medium was measured as an indicator of nitric oxide production using Griess reagent (Sigma-Aldrich, Canada). Briefly, cell culture medium was mixed with Griess reagent at a 1 to 1 ratio, incubated at room temperature for 30min in the dark and measured at 540nm absorbance on a UV-Vis microplate reader. Fresh culture medium was used as a blank in every experiment. The quantity of nitrite was determined by regression analysis using a sodium nitrite standard curve.

5.3.6 Arginase activity assay

Arginase activity was evaluated using the Arginase Activity Assay Kit (MAK112-1KT, Sigma-Aldrich) according to the manufacturer's instructions. Briefly, RAW 264.7 macrophages were lysed using lysis buffer containing 10mM Tris-HCl pH 7.4, 1µM pepstatin A, 1µM leupeptin and 0.4% Triton X-100. Samples were centrifuged at 13,000×g for 10min to remove insoluble contents and incubated in a covered 96-well plate with substrate buffer for 2h at 37 °C. The urea produced from arginase activity reacts with the substrate buffer to generate a coloured product. The Urea Reagent was added to the plate and incubated for 60min at room temperature to stop the reaction. Finally, the 96-well plate was measured at 430nm absorbance on a UV-Vis microplate reader. The arginase activity was determined per the following equation:

$$\frac{(A_{430})\text{sample} - (A_{430})\text{blank}}{(A_{430})\text{standard} - (A_{430})\text{water}} \times \frac{(1\text{mM} \times 50\mu\text{l} \times 10^3)}{(40\mu\text{l} \times 120\text{min})}$$

5.3.7 Subcellular fractionation

Cellular compartments are sequentially extracted by incubating cells with the Subcellular Protein Fractionation Kit (Millipore) for Cultured Cells according to the manufacturer's protocol. Cells were trypsinized and collected by centrifugation at 500 x g for 5 min using IEC Micromax RF Refrigerated Microcentrifuge. The cell pellet was washed once with ice-cold PBS and let dry. Extraction buffers were added sequentially to generate five cellular fractions: cytoplasmic, membrane (from plasma, ER, Golgi, mitochondria, and vesicular storage units), soluble nuclear, chromatin-bound and cytoskeletal. The extracts obtained were analyzed by western blotting with the corresponding controls: cytoplasm, membrane, and soluble nuclear contents.

5.3.8 Immunofluorescence and scanning confocal microscopy

RAW 264.7 cells were cultured on chamber slides at final confluency of 60%. Cells were fixed with 100% acetone for 3min on ice, washed with PBS, and permeabilized with 0.3% Triton X-100 in PBS for 2min. After three PBS washes, cells were blocked with 5% BSA in PBS for 1h at room temperature and incubated with the primary antibody in 1% BSA for 1h at 37 °C (PHB 1:1000, PHB2 1:1000, Cell Signaling). Cells were washed in PBS three times and then incubated in secondary antibody in 1% BSA for 2h at room temperature in the dark (rabbit anti-mouse 1:1000, Invitrogen). Again, cells were washed in PBS three times and stained with DAPI for 30min (1:5000). Finally, the cells were washed in PBS and the slides were mounted and viewed using a fluorescence microscope (Zeiss Axioskop, Germany). Images were further processed using ImageJ to adjust brightness/contrast and size/resolution.

5.3.9 RT-qPCR

Total RNA isolation was performed using TRIzol reagent (Sigma) according to the manufacturer's protocol. RNA was quantified by Nanodrop and 500ng was reverse transcribed

using First-Strand Synthesis System. The synthesized cDNA was then subject to RT-qPCR using SYBR Green Universal Mix (Life Technologies Inc. Canada) as per the manufacturer's protocol using 18S as an internal control. Gene-specific primer sequences used were as follows:

Table 5.1. qRT-PCR primer sequences used.

Name of Gene	**Sequences**
Il1b	F: AACTCAACTGTGAAATGCCACC R: CATCAGGACAGCCCAGGTC
Il6	F: TACTCGGCAAACCTAGTGCG R: GTGTCCCAACATTCATATTGTCAGT
Nos2	F: TTCCATGCTAATGCGAAAGG R: GCTCCTCTTCCAAGGTGCTT
Phb	F: GATTCCGTGGCGTACAGGA R: GCGGCAGTCAAAGATAATTGGTT
Phb2	F: ATCCGTGTTCACCGTGGAAG R: CCCGAATGTCATAGATGATGGG
Fabp4	F: AAGGTGAAGAGCATCATAACCCT R: TCACGCCTTTCATAACACATTCC
Arg1	F: CTCCAAGCCAAAGTCCTTAGAG R: AGGAGCTGTCATTAGGGACATC
Cd206	F: GGGCTTACGGTGAACCAAAT R: TGTCTTGTGGAGCAGGTGTG
Il10	F: GCTCTTACTGACTGGCATGAG R: CGCAGCTCTAGGAGCATGTG

5.3.10 Cytokine measurements

Various cytokines in the conditioned media were measured by Bio-Plex 200 multiplex suspension array system using murine cytokine panel (Bio-Rad, CA) as per manufacturer's protocol.

5.3.11 Western immunoblotting

Primary and secondary antibodies used in this study were obtained from Cell Signaling Technology (Danvers, MA, United States) unless mentioned otherwise. In brief, total cell lysates containing equal amount of proteins (~30 μg/lane) were separated by SDS-PAGE and subsequently analyzed by immunoblotting using protein-specific primary antibody and an HRP-conjugated secondary antibody [Table 5.2]. Finally, immunodetection was performed using an enhanced chemiluminescence kit (GE Healthcare, Mississauga, ON).

Table 5.2. Primary and secondary antibodies used.

Antibody name	Host	Dilution	Company
PHB	Rabbit	1:2000	CST
PHB2	Rabbit	1:2000	CST
Erk1/2	Rabbit	1:1000	CST
pErk1/2 T202/Y204	Rabbit	1:1000	CST
Akt (Pan)	Rabbit	1:1000	CST
pAkt S473	Rabbit	1:1000	CST
JAK1	Rabbit	1:1000	CST
pJAK1 Y1034/Y1035	Rabbit	1:1000	CST
STAT1	Rabbit	1:1500	CST
pSTAT1 Y701	Rabbit	1:1500	CST

STAT6	Rabbit	1:1000	CST
pSTAT6 Y641	Rabbit	1:1000	CST
IKKα/β	Rabbit	1:1000	CST
pIKKα/β S176/S177	Rabbit	1:1000	CST
α/βTubulin	Rabbit	1:3000	CST
βActin	Rabbit	1:3000	CST
Anti-rabbit HRP-linked	-	1:5000	CST
COX-IV	Mouse	1:1000	Abcam
Histone H3	Mouse	1:4000	Abcam
Goat-anti-mouse HRP-linked	-	1:5000	CST
Goat-anti-rabbit FITC-conjugated IgG	-	1:1500	CST

5.3.12 Cellular energetics

RAW 264.7 macrophages were seeded in the XF24-well assay plate (Seahorse Bioscience). After transfection and stimulation, macrophages were washed 3 times with assay buffer and incubated for an hour for measurements in oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). For OCR measurements, assay buffer was supplemented with 5 mM D-glucose and 1mM sodium pyruvate, pH 7.4 at 37 °C. Macrophages were treated with mitochondria perturbing agents: 1 µM of oligomycin, 1 µM of FCCP and 1 µM of rotenone/antimycin A sequentially. For ECAR measurements, assay buffer containing GlutaMax pH 7.4 at 37 °C was used without supplementation. Macrophages were treated with 1 µM of L-glucose, 1 µM of FCCP and 1 µM of deoxy-glucose sequentially. All experiments were performed in triplicates on the XF24 Seahorse extracellular flux analyzer (Agilent, Santa Clara, CA).

5.3.13 Statistical analysis

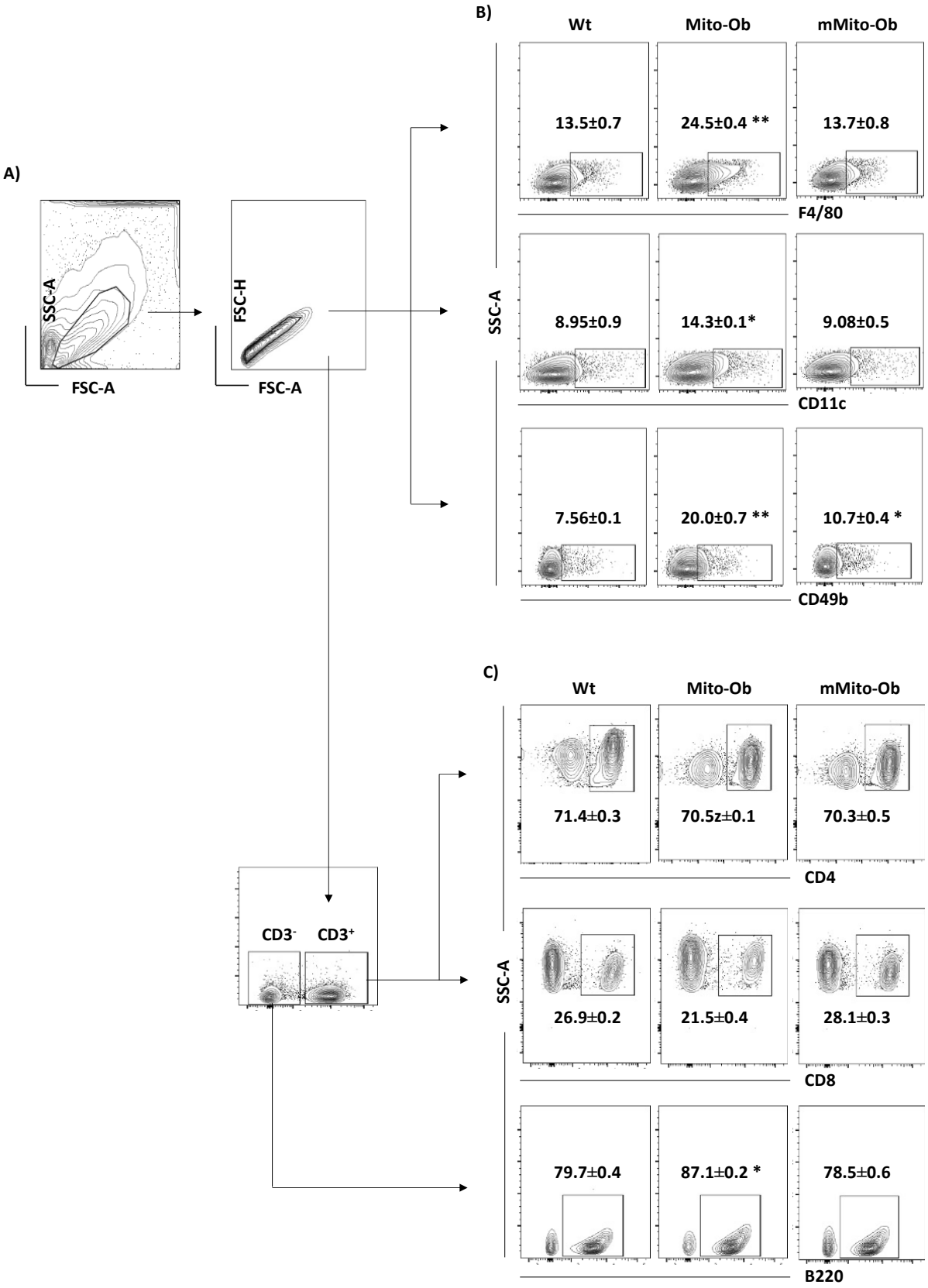
The data were analyzed using Prism 6.0 (La Jolla, CA) and presented as mean ± SEM. Two-tailed student's unpaired *t*-tests were performed to compare PHB/mPHB transfected cells or PHB knockdown cells and respective control cells (vector-transfected or scrambled shRNA transfected as applicable). Dunnett's *t*-tests were performed in which comparisons were made between multiple groups with a control group. *P*-values < 0.05 were considered significantly different.

5.4 Results

5.4.1 Male Mito-Ob mice have an increased innate cell population compared with wild-type and mMito-Ob mice

Previously, we found that male mMito-Ob mice developed immune-related abnormalities over time [7,26], including lymph node tumours and splenomegaly or insulitis in a context-dependent manner, which suggest that a loss of Tyr-114 phosphorylation in PHB may have contributed to the dysregulation. In addition, we have found that Mito-Ob and mMito-Ob mice have different serum cytokine profiles [7]. The lymph nodes and spleen are secondary lymphoid organs where immune cells accumulate. To obtain a more comprehensive view of immune cell content, we performed flow cytometry to assess the abundance of key immune cell populations in the spleens of 24-week-old male mice [Figure 5.1A], where the immunophenotype was first observed [7]. Compared to the control wild-type mice, the innate cell population analyzed (macrophages, dendritic cells, and NK cells) increased significantly in Mito-Ob mice, while they remained similar in numbers in mMito-Ob mice [Figure 5.1B]. In the adaptive immune cell population, B cells were markedly increased only in the Mito-Ob mice, while the T cell population remained similar in all three genotypes

[Figure 5.1C]. These intriguing results suggested that overexpression of PHB may have mobilized distinct immune cell populations and provided protection against immunological dysregulation, whereas loss of Tyr-114 phosphorylation in Y114F-PHB (mPHB) led to immune dysregulation suggesting its involvement in PHB's role in immune cell functions. Since the *Fabp4* gene is selectively expressed in monocytic cells [22] and Mito-Ob and mMito-Ob mice display differences in their dysregulation, we proceed to focus on PHB in macrophages at the cellular level.


A)
SSC-A
FSC-A
FSC-H
FSC-A
B)
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mMito-Ob
13.5±0.7
24.5±0.4 **
13.7±0.8
F4/80
SSC-A
8.95±0.9
14.3±0.1*
9.08±0.5
CD11c
7.56±0.1
20.0±0.7 **
10.7±0.4 *
CD49b
CD3⁻
CD3⁺
C)
Wt
Mito-Ob
mMito-Ob
71.4±0.3
70.5z±0.1
70.3±0.5
CD4
SSC-A
26.9±0.2
21.5±0.4
28.1±0.3
CD8
79.7±0.4
87.1±0.2 *
78.5±0.6
B220

Figure 5.1. Spleen immunophenotyping of wild-type (Wt), Mito-Ob and mMito-Ob mice at 24 weeks. A) Single-cell suspensions of the splenocytes were assessed for immune cells by flow cytometry utilizing an established gating strategy. B) Frequencies of total macrophages ($F4/80^+$), dendritic cells ($CD11c^+$) and natural killer cells ($CD49b^+$) in the spleen of Wt, Mito-Ob and mMito-Ob mice. C) Frequencies of total $CD4^+$ T cells, $CD8^+$ T cells and B cells ($B220^+$) in the spleen of Wt, Mito-Ob and mMito-Ob mice. Results are presented from 2 independent sets of experiments ($n = 8$ mice per experimental group) with similar results. $^*P < 0.05$; $^{**}P < 0.01$ compared with the Wt control group. FSC-A: Forward scatter area; SSC-A: Side scatter area; FSC-H: Forward scatter height.

5.4.2 PHB protein expression does not change during macrophage polarization

First, we wanted to determine whether PHB protein expression is altered in differentially activated macrophages, so a 24-hour time-course analysis was conducted with the M1 (LPS) and M2 (IL-4) stimuli in RAW 264.7 macrophages. Macrophage polarization was apparent by morphological change under light microscopy [Figure 5.2A]. First, different concentrations of each stimulus were tested to examine potential dosage influence, but there was no effect on the PHB protein level [Figure 5.2B]. Thereafter, 10 ng/ml LPS and 20 ng/ml IL-4 were selected for subsequent studies [24,25]. At the protein level, macrophage stimulation routinely upregulated representative signalling molecules in the M1 and M2 phenotypes, but there was no change in PHB protein level in all the stimuli examined [Figure 5.2C, D]. In M1 macrophages, there was a routine upregulation of *Il1b*, *Il6*, and *Inos* gene transcripts after LPS stimulation [Figure 5.2E]. In M2 macrophages, *Arg1*, *Il10* and *Cd206* gene transcripts have peaked at various time points after IL-4 stimulation [Figure 5.2F]. Interestingly, the *Phb* gene transcripts and its commonly associated partner, *Phb2*, were both downregulated over the 24-hour period only in the M1 macrophages [Figure 5.2E]. This was not reflected at the protein level indicating a context-dependent relationship between *Phb* mRNA and protein levels in macrophages.

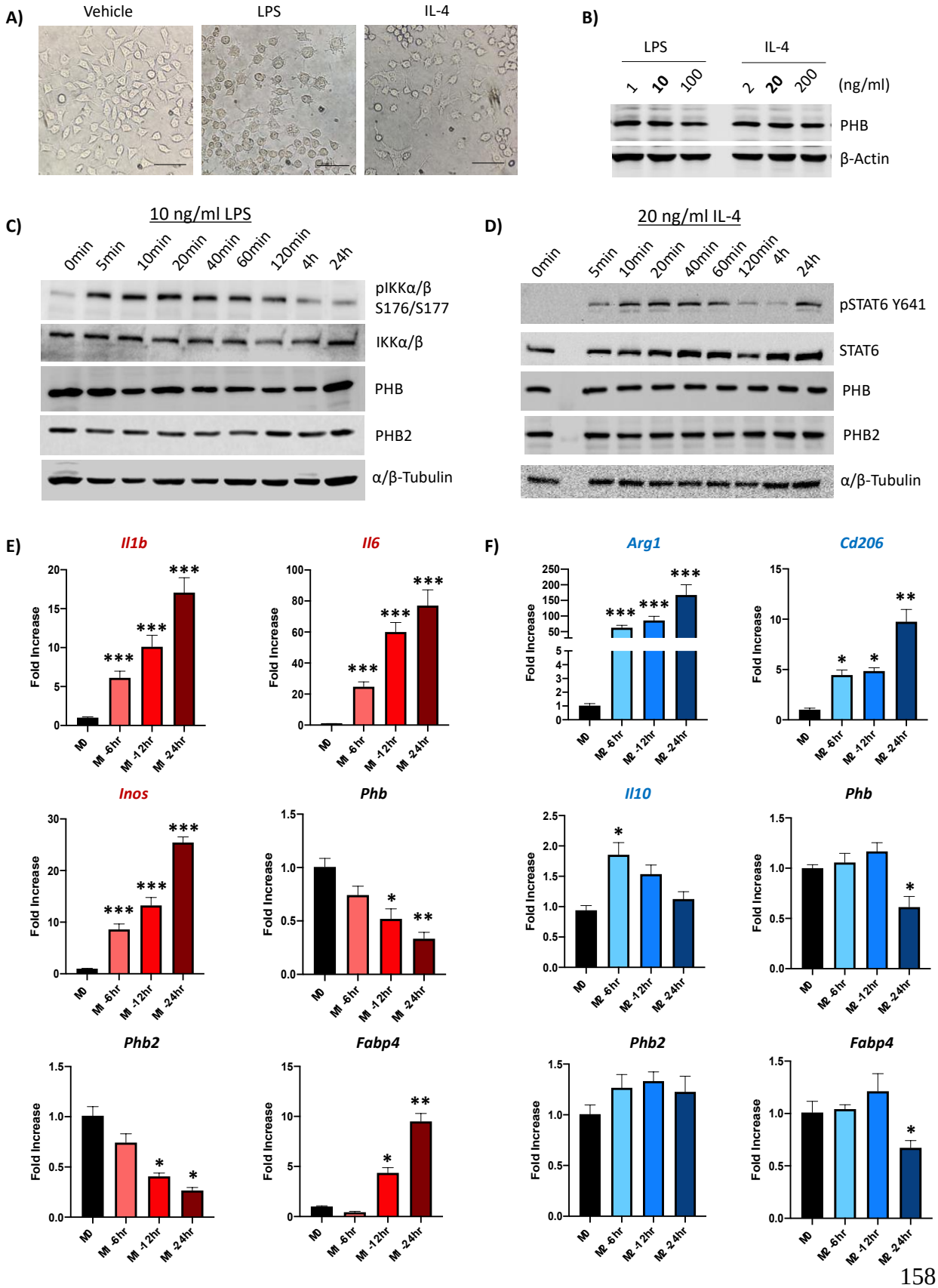

A)
Vehicle
LPS
IL-4
B)
LPS
IL-4
1
10
100
2
20
200
(ng/ml)
PHB
β-Actin
C)
10 ng/ml LPS
0min
5min
10min
20min
40min
60min
120min
4h
24h
pIKKα/β S176/S177
IKKα/β
PHB
PHB2
α/β-Tubulin
D)
20 ng/ml IL-4
0min
5min
10min
20min
40min
60min
120min
4h
24h
pSTAT6 Y641
STAT6
PHB
PHB2
α/β-Tubulin
E)
Il1b
Il6
Inos
Phb
Phb2
Fabp4
Fold Increase
M0
M1 -6hr
M1 -12hr
M1 -24hr
F)
Arg1
Cd206
Il10
Phb
Phb2
Fabp4
Fold Increase
M0
M2 -6hr
M2 -12hr
M2 -24hr

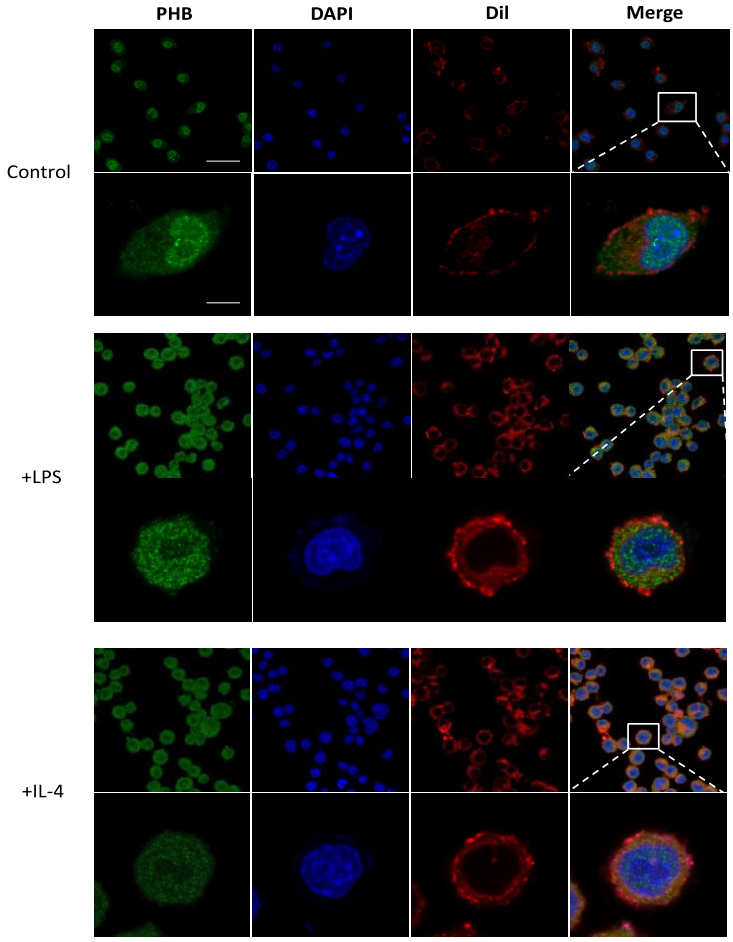
Figure 5.2. Gene transcript and protein expression levels of PHB in RAW 264.7 macrophages. RAW 264.7 cells were stimulated for up to 24 h. M1 macrophages were stimulated with 10 ng/ml of LPS and M2 macrophages were stimulated with 20 ng/ml of IL-4 unless otherwise stated. A) Representative light microscope images of the M1 and M2 macrophages 24 h post polarization. Scale bar = 40 μm. B) Different concentrations of LPS and IL-4 were used to stimulate RAW 264.7 macrophages for 24 h and PHB expression was examined using immunoblotting. C and D) PHB protein level and the signature signalling molecules of M1 and M2 polarized states were examined using immunoblotting. α/β-Tubulin or β-Actin was used as a loading control. E and F) PHB gene transcript level and the signature gene expression of M1 and M2 polarized states were examined using RT-qPCR. Data are presented as mean ± SEM (n = 4); *P < 0.05, **P < 0.01, and ***P < 0.001 in reference to vehicle-treated macrophages (M0).

5.4.3 PHB translocates from the nucleus upon macrophage polarization

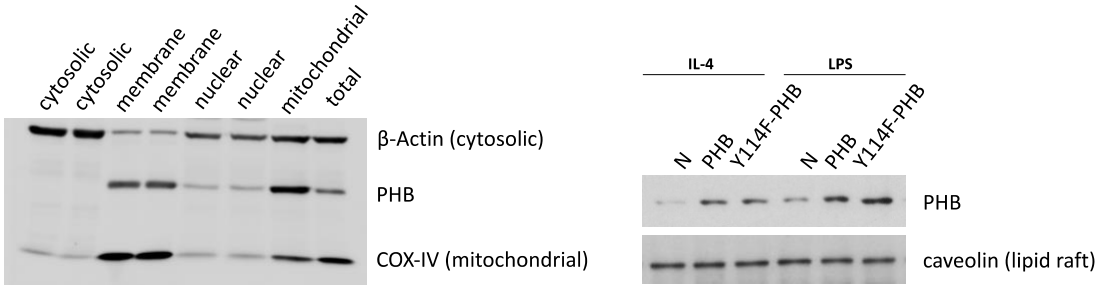
It is well known that PHB is found in multiple subcellular compartments, including the mitochondria, nucleus, and plasma membrane [7,8]. Immunofluorescence confocal scanning microscopy revealed that PHB is predominately localized to the nucleus of untreated macrophages [Figure 5.3A]. After both LPS and IL-4 stimulations, PHB was found outside the nucleus (which was more apparent in response to LPS stimulation) suggesting possible subcellular translocation. Next, we performed subcellular fractionation and found that PHB was associated with the membrane system, including the nuclear membrane, cell membrane and mitochondrial membrane [Figure 5.3B]. PHB is a member of the SPFH superfamily, which has been found in lipid raft micro-domains in diverse cellular membranes [27]. Lipid rafts play diverse roles in the cell, one of which is to compartmentalize cellular functions within the plane of biological membranes [28]. To further measure the cell membrane PHB, we probed for PHB by flow cytometry with and without cell permeabilization. During a 24-hour time course, both PHB surface and total levels increased correlatively and significantly after LPS stimulation [Figure 5.3C] suggesting that PHB translocates from the nucleus upon LPS-induced macrophage polarization. No change in PHB

level was observed after IL-4 stimulation [data not shown]. In addition, we examined PHB levels in the membrane lipid raft fraction in macrophages [20]. A difference was found in PHB and mPHB levels in comparison with the respective control group (i.e., IL-4 and LPS treatment) [Figure 5.3B]. In addition, PHB and mPHB levels were relatively higher in the membrane lipid raft fraction of LPS-treated cells when compared with IL-4-treated cells [Figure 5.3B].

A)


PHB
DAPI
Dil
Merge
Control
+LPS
+IL-4

B)


cytosolic
cytosolic
membrane
membrane
nuclear
nuclear
mitochondrial
total
β-Actin (cytosolic)
PHB
COX-IV (mitochondrial)
IL-4
LPS
N
PHB
Y114F-PHB
N
PHB
Y114F-PHB
PHB
caveolin (lipid raft)

C)

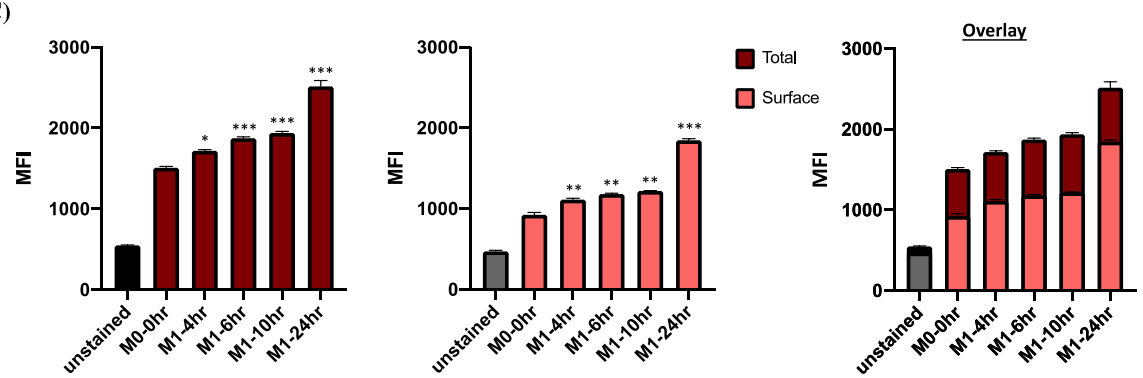

MFI
3000
2000
1000
0
unstained
M0-0hr
M1-4hr
M1-6hr
M1-10hr
M1-24hr
Total
Surface
Overlay

Figure 5.3. PHB localization after 24h of LPS (10ng/ml) or IL-4 (20ng/ml) stimulation in RAW 264.7 macrophages. A) Representative images were generated using immunofluorescence confocal scanning microscopy. Three antibodies were used against PHB (green), DAPI-nuclei (blue), and 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil)-membrane (red). Upper scale bar: 45 μm; lower scare bar: 10 μm. B) Left panel: Unstimulated macrophages were fractionated into cytoplasmic, membrane, and soluble nuclear fractions. Isolated mitochondria and total cell lysate were included for comparison. COX-IV: Mitochondrial marker; β-Actin: Cytosolic marker. Right panel: Immunoblot showing PHB levels in lipid raft fraction with/without stimulation; caveolin: Lipid raft marker. C) Surface and total PHB level in RAW 264.7 macrophages up to 24 h of LPS (10 ng/ml) stimulation. Change in PHB expression level was analyzed by flow cytometry and expressed as mean fluorescence intensity (MFI). Total and surface PHB were labelled with anti-PHB primary antibody and fluorescein isothiocyanate (FITC)-conjugated secondary antibody in permeabilized and intact macrophages, respectively.

5.4.4 Overexpression of PHB or mPHB in macrophages differently alters cytokine production

To examine the cellular pathways governing the functions of macrophages in Mito-Ob and mMito-Ob mice, we overexpressed PHB or mPHB in RAW 264.7 macrophages and subsequently polarized them to the M1 or M2 phenotype. Transfection efficiency was validated at the gene transcripts and protein levels [Figure 5.4A, B]. In M1 macrophages, LPS stimulation routinely increased pro-inflammatory gene transcripts *Il1b* and *Nos2*, and the production of cytokines IL-1β, IL-6, IL-12 and TNFα, which were consistent with the well-characterized M1 phenotype. However, PHB overexpression significantly reduced the synthesis and production of these cytokines [Figure 5.4C]. Whereas, overexpression of mPHB did not share this reduction, instead, it showed similar levels as the control condition. The reversal of PHB-induced cytokine suppression under mPHB overexpression suggests that the phosphorylation site plays a critical role in orchestrating cytokine synthesis and production. Since almost all cytokines analyzed were downregulated in PHB-overexpressed M1 macrophages, we suspect that the regulation by PHB may have occurred at the early stages of M1 polarization.

On the other hand, overexpression of PHB in M2 macrophages led to a significant increase in IL-10 [Figure 5.4C], whereas an opposite effect was observed in mPHB overexpressing cells [Figure 5.4C] suggesting the involvement of Tyr-114 site in PHB in its anti-inflammatory function. In aggregate, this data suggests that PHB plays a context-dependent anti-inflammatory role in macrophages (i.e., suppresses pro-inflammatory function and promotes anti-inflammatory function), which involves Tyr-114 residue.

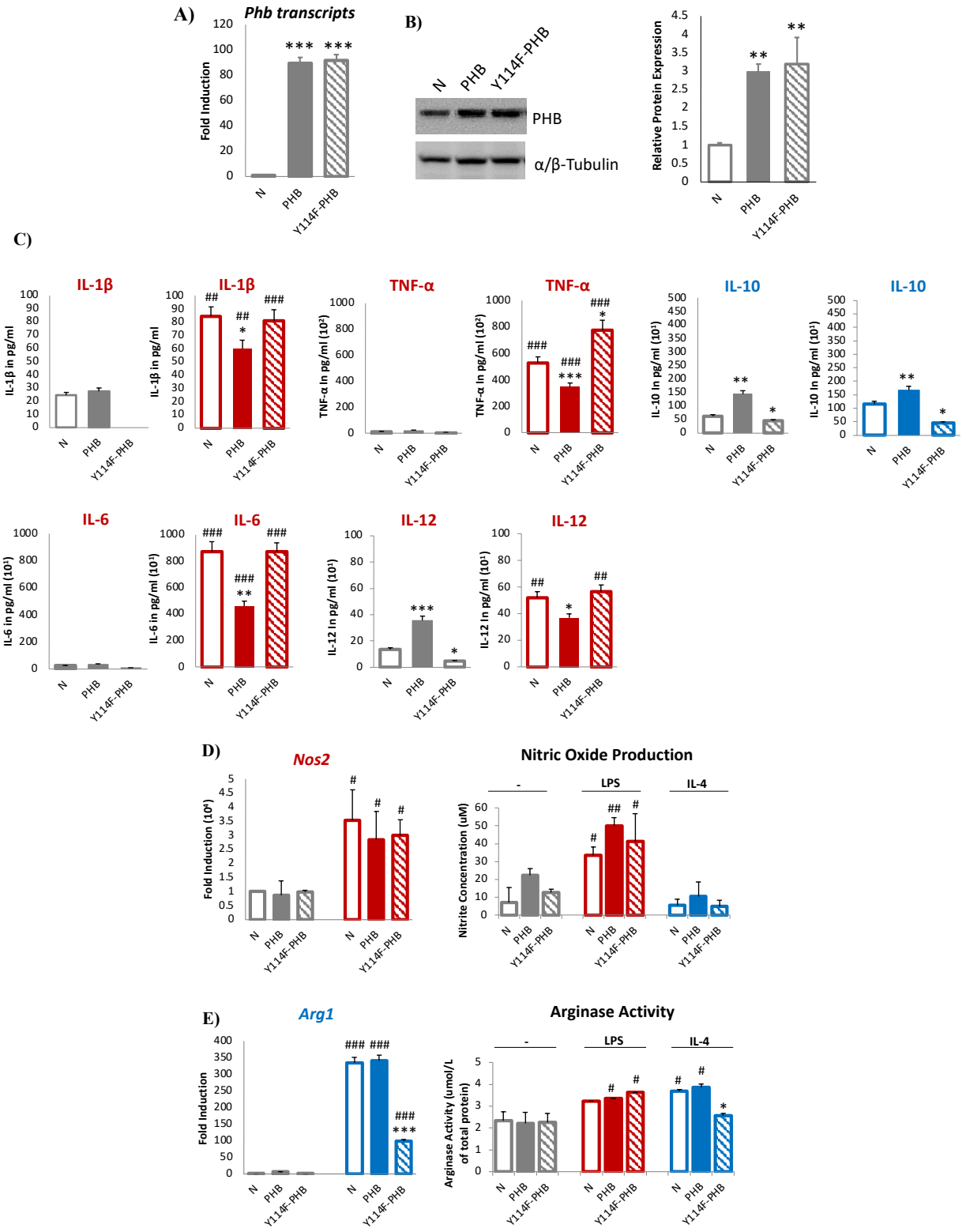

A)
Phb transcripts
Fold Induction
N
PHB
Y114F-PHB
B)
PHB
α/β-Tubulin
Relative Protein Expression
C)
IL-1β
IL-1β in pg/ml
TNF-α
TNF-α In pg/ml (10²)
IL-10
IL-10 In pg/ml (10¹)
IL-6
IL-6 in pg/ml (10¹)
IL-12
IL-12 In pg/ml (10¹)
D)
Nos2
Fold Induction (10⁶)
Nitric Oxide Production
Nitrite Concentration (uM)
-
LPS
IL-4
E)
Arg1
Fold Induction
Arginase Activity
Arginase Activity (umol/L of total protein)

Figure 5.4. Functional output of RAW 264.7 macrophages after 24 h of LPS (10 ng/ml) or IL-4 (20 ng/ml) stimulation under PHB or Y114F-PHB overexpression. Cells were transfected with PHB (solid bar) or Y114F-PHB (stripe bar); Gray colours = unstimulated; Red colours = LPS-stimulated; Blue colours = IL-4 stimulated. N = vector-transfected control. Data are presented as mean ± SEM (n = 3). (A) RT-qPCR confirmed the over-expression of PHB or Y114F-PHB gene transcripts. (B) Western quantification confirmed overexpression of PHB or Y114F-PHB protein. (C) Cytokine secretions in RAW 264.7 macrophages. Signature M1 and M2 markers were selected and examined using RT-qPCR. (D) The quantity of nitrites (NO_2^-) in the cell medium was measured as an indicator of nitric oxide (NO) production, a signature functional output of M1 macrophage. (E) The Arginase activity of cell lysates was analyzed as a signature functional output of M2 macrophages. Results were normalized to cell viability by dividing activity by viability. Data are presented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001 by Student's t-test between N and PHB or Y114F-PHB; #P < 0.05, ##P < 0.01, and ###P < 0.001 by Student's t-test between unstimulated and M1 or M2 polarized state.

5.4.5 Overexpression of mPHB in macrophages alters arginase activities but not nitric oxide production

Arginine metabolism can be used as a functional characterization to differentiate the M1 and M2 phenotypes [29]. M1 macrophages upregulate inducible nitric oxide synthase (iNOs) through the STAT1 pathway upon LPS stimulation, which metabolizes arginine to nitric oxide (NO) and citrulline. NO plays a key role in anti-microbial action, cytotoxicity and immune regulation. M2 macrophages are characterized by the expression of arginase 1 (ARG1) through the STAT3/6 pathway, which hydrolyzes arginine to ornithine and urea. In M1 macrophages, iNOs expression and production appear to be unaffected by PHB and mPHB overexpression [Figure 5.4D], which suggests that the effect of PHB is likely not mediated by this pro-inflammatory axis. In M2 macrophages, both control and PHB overexpression enhanced the expression and activity of ARG1 [Figure 5.4E]. Under Y114F mutation in PHB, however, both ARG1 expression and activities were significantly decreased [Figure 5.4E]. This observation prompted further examination of upstream signalling pathways of ARG1 in hopes of narrowing down the site of PHB function.

5.4.6 Overexpression of mPHB in M2 macrophages inhibits STAT6 phosphorylation

JAK1-STAT6 is the major downstream kinases at the IL-4 receptor complex in macrophages, which are phosphorylated at Tyr-1034/1035 and Tyr-641, respectively. Previously, researchers have found that phosphorylation of PHB at Tyr-114 and Tyr-259 by Lyn kinase is required for the formation of the IgE receptor complex, FcεRI, in mast cells [19]. To explore a potentially similar role of PHB in the IL-4 receptor complex, we examined the phosphorylation status of JAK1 and STAT6 while overexpressing PHB or mPHB in M2 macrophages. PHB and mPHB overexpression induced JAK1 phosphorylation in control macrophages, but M2

macrophages did not show a significant change in JAK1 phosphorylation compared with control macrophages [Figure 5.5B]; however, an increasing trend was observed in mPHB expressing M2 macrophages. Robust phosphorylation of STAT6 at Tyr-641 was observed in control M2 macrophages in response to IL-4 stimulation, which was relatively lower in PHB overexpressing cells and substantially suppressed in mPHB overexpressing macrophages [Figure 5.5C]. The result provided evidence that phosphorylation of PHB at Tyr-114 is required for STAT6 phosphorylation at Tyr-641 near the IL-4 receptor complex.

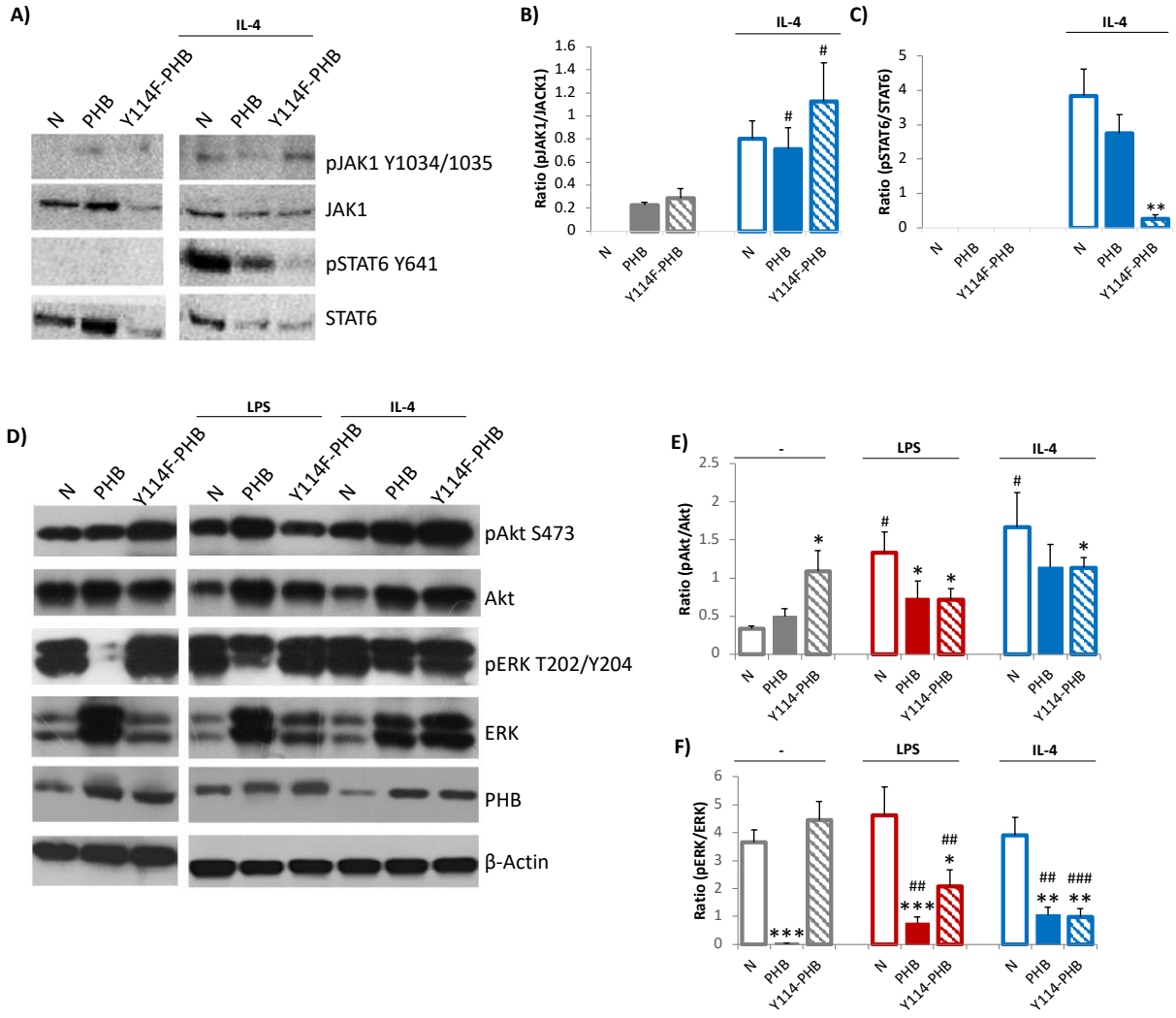


Figure 5.5. Cell signalling molecules in RAW 264.7 macrophages after LPS or IL-4 stimulation under PHB or Y114F-PHB overexpression. Cells were transfected with PHB (solid bar) or Y114F-PHB (stripe bar); Gray bars = unstimulated; Red bars = LPS-stimulated; Blue bars = IL-4 stimulated. N = vector-transfected control. Data are presented as mean ± SEM (n = 3). A) Representative immunoblots of JAK and STAT6 phosphorylation in unstimulated and M2 macrophages. B) and C) Ratios of pJAK1/JAK1 and pSTAT6/STAT6 were analyzed through western quantification. D) Akt and ERK phosphorylation in M1 and M2 polarized states were examined. β-Actin was used as a loading control. E and F) Ratios of pAkt/Akt and pERK/ERK were analyzed through western quantification. *P < 0.05, **P < 0.01, and ***P < 0.001 by Student's t-test between N and PHB or Y114F-PHB; #P < 0.05, ##P < 0.01, and ###P < 0.001 by Student's t-test between unstimulated and M1 or M2 polarized state. Data are presented as mean ± SEM (n = 3).

5.4.7 PHB differentially affects the Akt and ERK1/2 pathways in macrophages

A number of signalling pathways in the functional plasticity of macrophages are also modulated by PHB [30]. For instance, Akt is associated with the metabolic switches in macrophages in response to upstream stimuli, and Tyr-114 phosphorylation at PHB negatively regulates Akt signalling in other cell types [11,31]. Moreover, the MEK/ERK pathway is involved in mediating the effects of macrophage polarization, and Tyr-114 and Thr-258 in PHB have been reported to have a role in ERK regulation [32]. These signalling pathways are known to crosstalk and exert a negative regulation on each other [30], and previously we have found the phosphorylation of PHB at Tyr-114 and Thr-258 have a similar effect on Akt and ERK regulation in metabolic cell/tissue types [33]. Therefore, we examined the activity level of both Akt and ERK in response to M1 and M2 macrophages overexpressing PHB and Y114F-PHB. The phospho-Akt (pAkt) level was significantly higher in Y114F-PHB overexpressing macrophages [Figure 5.5D]. Upon stimulation by both M1 and M2 stimuli, the pAkt level increased significantly compared to the unstimulated state, which was relatively lower under PHB or Y114F-PHB overexpression in response to LPS and IL-4 [Figure 5.5E]. When looking at ERK phosphorylation, PHB overexpression almost completely suppressed ERK phosphorylation in the control group, whereas such an effect was not observed in Y114F-PHB overexpressing cells [Figure 5.5E]. In M1 macrophages, a significant decrease in the phospho-ERK (pERK) level was observed in PHB and Y114F-PHB overexpressing cells (compared with control cells); however, a difference in pERK and total ERK was apparent between them [Figure 5.5E] suggesting a role of PHB in the regulation of ERK stability and function. However, such a difference in the stability and phosphorylation of ERK was not observed in M2 macrophages [Figure 5.5E].

5.4.8 PHB knockdown or mPHB overexpression in macrophages inhibits mitochondrial respiration

In addition to membrane signalling, PHB has been shown to play a crucial role in mitochondrial biology in many cell types [34]. It maintains the overall homeostasis of the mitochondria by stabilizing the respiratory complexes and mitochondrial DNA. For this reason, we investigated mitochondrial respiration in macrophages using the Seahorse XF analyzer. No significant difference was found in basal oxygen consumption rate (OCR) between transfection control and PHB-overexpressing cells [Figure 5.6A]. However, basal OCR was significantly reduced in cells that overexpressed Y114F-PHB compared with vector-transfected control and cells that overexpressed PHB [Figure 5.6B]. In contrast to overexpression, PHB knockdown by shRNA in macrophages led to a significant reduction in basal OCR in comparison with scrambled siRNA transfected control cells [Figure 5.6A]. Taken together, these data suggest that the phosphorylation of PHB at Tyr-114 has a role in the regulation of mitochondrial respiration in macrophages. No change in basal OCR in PHB overexpressing cells would imply that the role of PHB in mitochondrial respiration in macrophages might require modulation by upstream stimuli, which may involve the phosphorylation of PHB at Tyr-114 residue or potentially other residues, which are functionally related to Tyr-114 as we have reported in other cell types [10].

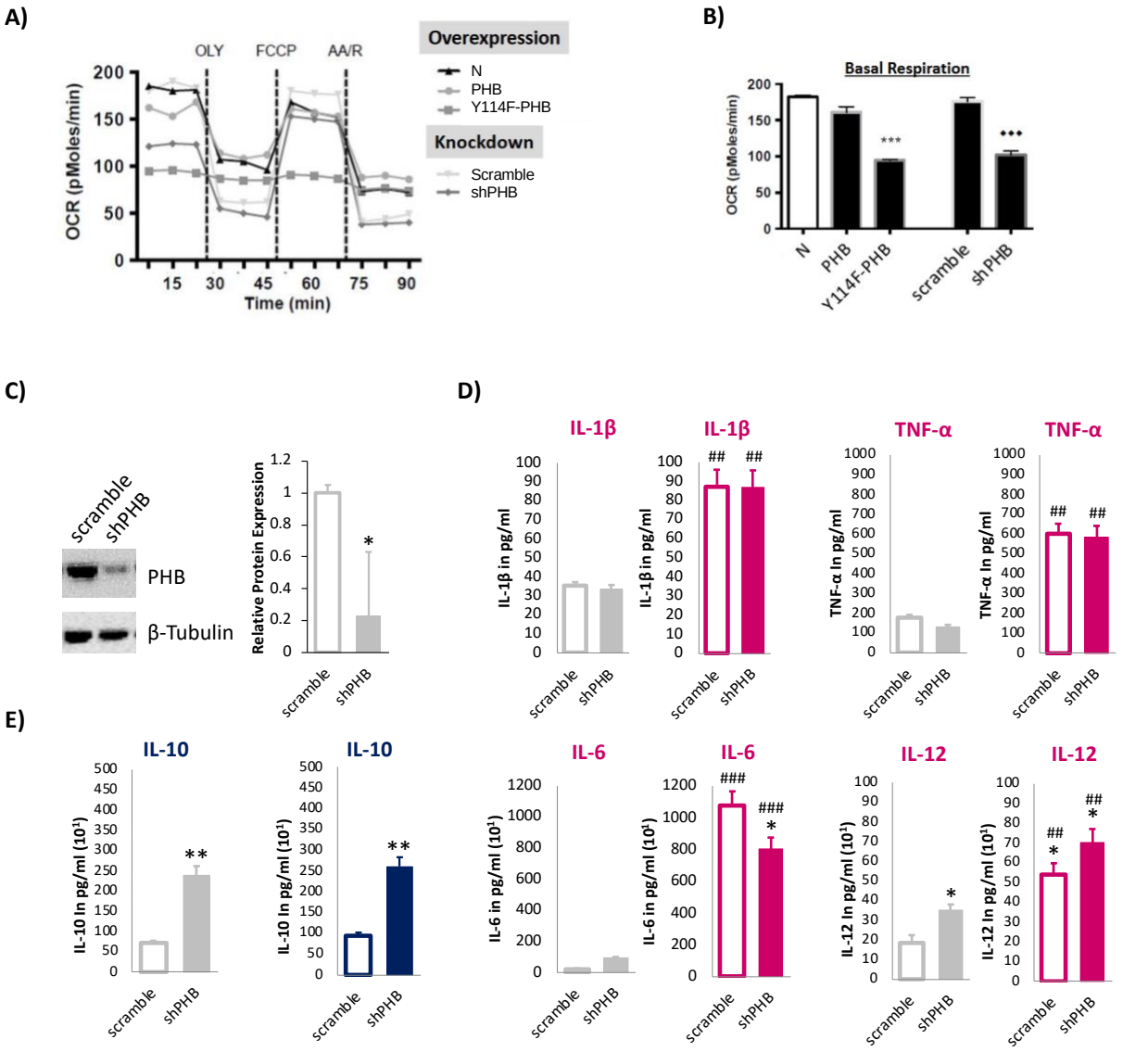


Figure 5.6. PHB knockdown in macrophages inhibits mitochondrial respiration and alters cytokine production. A) Line graph showing oxygen consumption rate (OCR) in macrophages transfected with PHB, Y114F-PHB and control (N) as determined by Seahorse metabolic flux analyzer. B) Histograms showing basal OCR in macrophages after PHB overexpression and knockdown. Data are presented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01 by Dunnett's t-test between control and Y114F-PHB. ##P < 0.01, ###P < 0.001 by Student's t-test between scrambled shRNA and PHB shRNA. OLY: Oligomycin; FCCP: Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone; AA/R: Antimycin/Rotenone. C) Western quantification confirmed the knockdown of PHB protein. D and E) Histograms showing proinflammatory and anti-inflammatory cytokine secretions in RAW 264.7 macrophages after PHB knockdown.

5.4.9 Knockdown of PHB in macrophages alters macrophage cytokine production

Next, we also examined the effect of PHB knockdown on the signature cytokines of M1 and M2 macrophages. PHB knockdown in macrophages seems to have a diverse effect on different cytokine production when compared with the control group [Figure 5.6D, E]. For example, IL-6 production was downregulated, IL-1β and TNFα showed no difference, whereas IL-12 and IL-10 production were upregulated. In M2 macrophages, the one marker analyzed, IL-10, was upregulated upon PHB knockdown.

5.5 Discussion

Macrophages are key modulators and effectors in immunity and metabolism. It is valuable to define various aspects of macrophage biology and better understand its role in health and disease. In this study, we have investigated the effects of LPS and IL-4 as representative M1 and M2 stimuli in PHB-manipulated macrophages [Figure 5.7]. The M1 stimuli are grouped according to their ability to induce prototypic inflammatory responses and markers, but their source, role, receptors and signalling pathways differ substantially [35]. LPS is the best-studied M1 macrophage signal, which leads to strong production of pro-inflammatory cytokines (e.g., IL-1β, IL-6, IL-12, and TNFα) and chemokines (e.g., CCL2, and chemokine ligand CXCL10 and CXCL11) [35]. A reduction in pro-inflammatory cytokine production from macrophages upon PHB overexpression suggests that PHB plays a role in the early pathways leading up to overall cytokine production. Similar to the M1 stimuli, there is a high degree of variation in M2 stimuli and their signalling cascades. The well-studied IL-4 exerts its action by binding to its receptor and activating the JAK1-STAT6 signalling cascade [35]. Previous studies have found that PHB is immunoprecipitated with phospho-STAT3 and has an anti-inflammatory role in intestinal epithelial cells [36,37]. The reduction in

STAT6 phosphorylation under Y114F-PHB overexpression suggests that PHB plays a crucial role in the anti-inflammatory effect of IL-4 in macrophages. Surprisingly, pJAK1 in PHB or Y114F-PHB overexpressing macrophages was detected in the absence of IL-4, which did not translate to STAT6 phosphorylation. We speculate that a relationship between JAK1 and PHB may exist beyond the JAK1-STAT6 signalling pathway, and it is associated with the level of PHB rather than PHB's phosphorylation status.

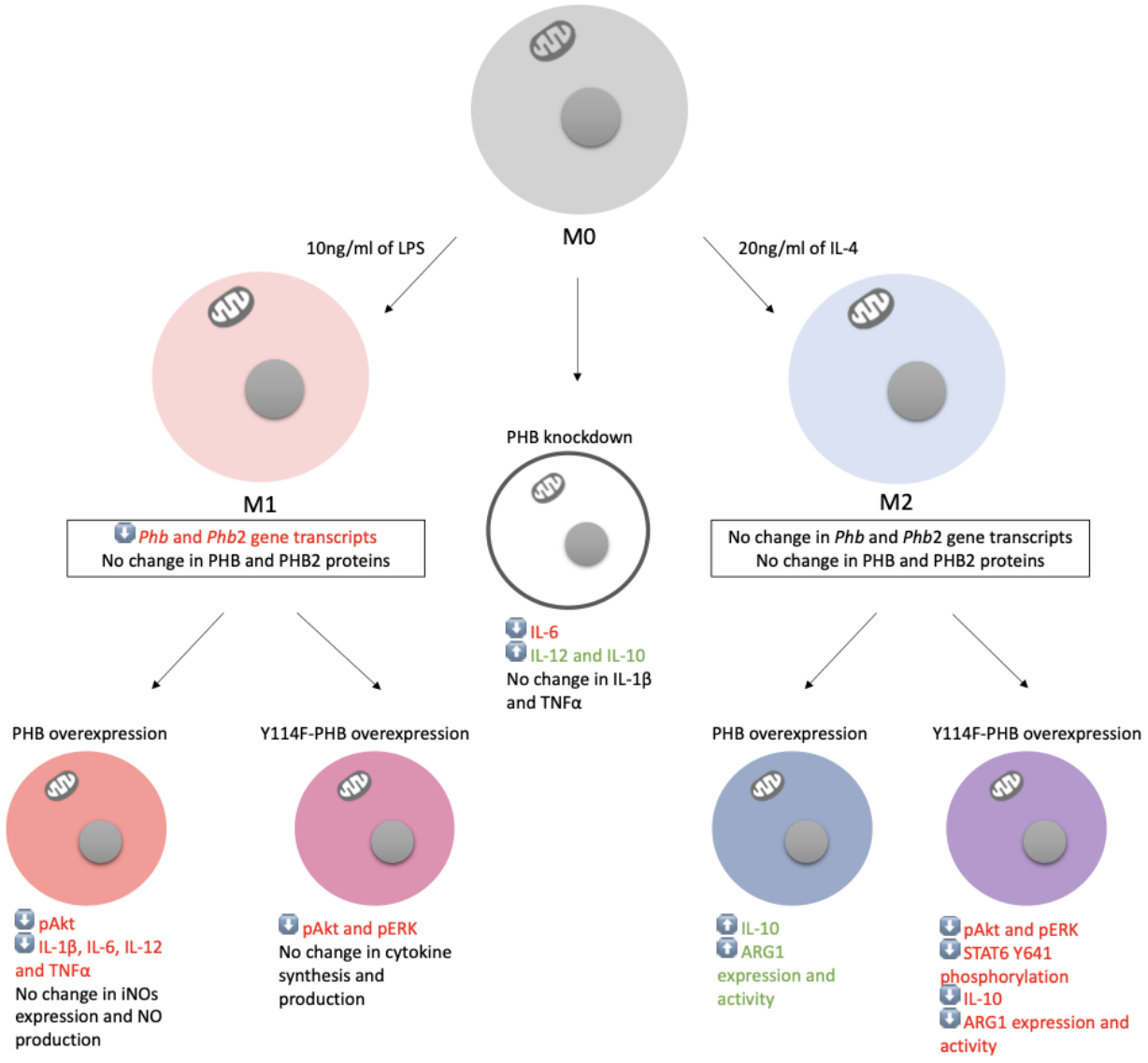


Figure 5.7. Schematic summary of PHB's role in the functional plasticity of macrophages. Naïve macrophages (M0) were stimulated with LPS or IL-4 to produce the M1 or M2 phenotype, respectively. Within each polarized state, the effects of PHB overexpression and Y114F-PHB overexpression were examined. PHB knockdown was also briefly examined in terms of cytokine production and mitochondrial respiration. The various effects are listed under each phenotype, which is compared directly to the phenotype above.

A number of cell signalling pathways are known to crosstalk and regulate each other, such as the PI3K/Akt and MEK/ERK signalling pathways. Phosphorylated PHB at multiple sites, including Tyr-114, Thr-258 and Tyr-259, often behave as an adaptor molecule in a number of cell signalling pathways [15]. For example, Tyr-114 phosphorylation of PHB facilitates the recruitment of SH2 domain-containing proteins [10], which can be found in a number of downstream effectors of the M1 and M2 stimuli (e.g., Src, Grb2, PLCγ, Shc, STATs, Tyk2) [38]. Thus, PHB may also be involved in a range of signalling pathways.

PHB is localized to different cellular compartments and has cell compartment-specific functions, which can influence macrophage biology independently or collectively. In this study, the observed change in macrophage signalling during activation is likely related to its role within the endomembrane system based on several pieces of evidence. First, PHB was previously identified in the phagosome membrane [39] and is found in the mitochondria, nucleus, and plasma membrane in macrophages under the unstimulated state in the study. Interestingly, PHB was visibly inside the nucleus before stimulation and translocated out of the nucleus during both M1 and M2 stimulations. Since the protein level of PHB remains unchanged, translocation provides an energy-efficient way for PHB to perform its pleiotropic functions in multiple organelles. Second, Y114F-PHB disrupted STAT6 phosphorylation under IL-4 stimulation. Given that STAT6 is one of the first kinases activated at the IL-4 receptor complex, the regulation by PHB more likely occurred early and near the plasma membrane. Similarly, in M1 macrophages, PHB overexpression systematically downregulated pro-inflammatory cytokines, which also implies early regulation in the signalling pathway of LPS. PHB may modulate the LPS pathway through another phosphorylation site, such as Thr-258, which have been reported to regulate each other [10,11], or through its reported suppression of NFκB translocation and activity [40–42]. Moreover,

macrophages undergo extensive metabolic reprogramming in response to diverse environmental cues [6]. A substantial change in the rate of oxygen consumption as a result of overexpressing Y114F-PHB suggests that loss of Tyr-114 phosphorylation behaves as a dominant negative in relation to mitochondrial respiration. Thus, the metabolic status of macrophages upon PHB manipulation may be related to mitochondrial function.

The nuclear function of PHB was not explored in this study, but others have discovered diverse nuclear functions of PHB in other cell types, including regulators of transcription factors, DNA modifying enzymes, cell cycle proteins, and RNA-binding proteins [43]. A concerted early and late regulation by PHB at the plasma membrane through phosphorylation and the nucleus through gene expression may not be ruled out. While the cell compartment-specific functions of PHB are not mutually exclusive, the precise contribution from each organelle in macrophage biology remains indistinct. The observed results can be extended to explain partly the immunophenotype observed in male Mito-Ob and mMito-Ob mice, including their cytokine and chemokine profiles [7]. Mito-Ob mice exhibited an increased innate cell population [7], including macrophages, which suggests that Y114F-PHB triggered the immune dysregulation. In Y114F-PHB macrophages, signalling pathways involving IL-4, Akt and ERK were disrupted. These pathways in the context of macrophages are critical in the activation and metabolism of M1/M2 polarization [44,45]. An imbalance of macrophage M1/M2 polarization is associated with various disease states. When the M1 macrophage activation becomes dominant, the prolonged activation can contribute to chronic inflammation, as observed in the mMito-Ob mice [46]. Other immune cells are also likely to be affected by PHB mutation since PHB is deemed an essential protein in many immune cell types [8,47]. Another aspect of the phenotypic difference in Mito-Ob mice and mMito-Ob mice may be

facilitated by sex differences, especially estrogens, which appear to play a substantial role in susceptibility to infectious diseases, in response to vaccination, and autoimmune diseases [48].

In summary, we demonstrated that PHB influences multiple aspects of macrophage biology, from activation to metabolism [Figure 5.7]. Its role in both the pro-inflammatory and anti-inflammatory responses likely occurred early on in a highly context-specific manner. PHB coordinates multiple cellular events during the activation process while macrophage undergoes drastic reprogramming and facilitates the activated state to perform its designated functional output [Figure 5.7], whether it be cytokine production or resolving inflammation. A similar role of PHB may be found in other immune cell types such as dendritic cells, B cells and T cells. In aggregate, our data support that PHB is a crucial regulator in the functional plasticity of macrophages.

5.6 References

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CHAPTER 6: DISCUSSION OF LIMITATIONS

PHB is one of the most highly conserved proteins across the phylogenetic tree [1]. Homologues of PHB are found in bacteria, archaea, and all higher eukaryotes. PHB is a member of the SPFH domain-containing superfamily and is found to be ubiquitously expressed in all cell types [2]. The functions of PHB are distinguished based on its cellular location. The significance of PHB in multiple locations within a cell is largely enigmatic, but it likely contributes to the pleiotropic property of PHB. Previous studies demonstrated that PHB is predominantly found in the mitochondria, with some studies using it as a housekeeping protein to identify the mitochondrial fraction in mouse cardiac tissues [3]. Mitochondrial PHB located on the inner mitochondrial membrane forms a multimeric complex with its homologue PHB2, which is required for mitochondrial protein scaffolding and stability. Consequently, this protein complex is an integral component of the mitochondrial membrane system and its dynamics. PHB is also found in other membrane systems, including on the plasma membrane and on trafficking vesicles (e.g., phagosomes). In fact, other members of the SPFH domain-containing protein superfamily, such as erlins, stomatins, and flotillins are found in the lipid rafts of various membrane systems, suggesting the essential role of this superfamily in maintaining lipid raft structure [4–6]. In particular, the hydrophobic region at the C-terminus of SPFH-domain proteins is capable of self-assembly and substantively contributes to the lipid raft formation [7]. Other than being membrane-bound, PHB is also found free in the cytosol and nucleus and plays strikingly diverse roles in signal transduction and gene regulation, respectively. The PHB in the nucleus is implicated in tumour suppression through its regulation of p53 expression upon apoptotic stimuli [8,9]. On the other end, the cytosolic PHB is a key signal transducer in multiple mechanisms, including the Ras-mediated MEK-ERK pathway. This pathway relay information between the cell membrane receptors and transcriptional

regulation through the phosphorylation of cytosolic effectors, including PHB, to influence cell proliferation and differentiation. PHB is found near or associated with cell membrane receptors at the start of the signalling transduction. Unlike mitochondrial PHB, cytosolic PHB is likely a peripheral protein that adheres temporarily to the biological membrane. This reversible attachment allows PHB to relocate for signalling and trafficking. It would be beneficial to determine whether PHB contains any known membrane domains (e.g., C1, C2, PH, FYVE, PX, ENTH, and BAR domains [10]).

Overexpression of PHB in transgenic animal models generally has an anti-inflammatory effect [11–13]. In our experimental animal models, similar results were observed in the female Mito-Ob and mMito-Ob mice with the added influence of sex difference. In male Mito-Ob mice, however, metabolic complications related to obesity were apparent, whereas loss of Tyr-114 phosphorylation in mMito-Ob mice led to immune dysregulation [14,15]. In many immune cells, PHB co-localizes with crucial surface receptors that are important for their activations [16–19]. In macrophages, a myriad of surface receptors is expressed under the resting state and induces signal transduction upon binding of their ligands. For example, the anti-inflammatory ligand, IL-4, leads to the phosphorylation and activation of the JAK1/STAT6 pathway. Although the current thesis has not fully elucidated PHB signalling transduction in macrophage activation and polarization, the data presented indicate that this mechanism is facilitated by PHB phosphorylation. Specifically, the Tyr-114 site on PHB acts as a major substrate of this regulatory system in macrophages. In this context, PHB phosphorylation at Tyr-114 appears to be an important factor that contributes to STAT6 phosphorylation. PHB also governs aspects of functional output during macrophage polarization. Overexpression of PHB in macrophage cell lines led to decreased proinflammatory and enhanced anti-inflammatory markers in M1 and M2 phenotypes, respectively. Experiments

designed to identify proteins which specifically associate with PHB in future studies will reveal insights with respect to the cellular role of this polarization pathway.

There are general limitations in the approach of PHB analysis in macrophages. The current thesis overlooked certain complexities to focus on the role of PHB under the two well-established polarized states of the macrophage cell line. These complexities lead to the limitations of the thesis, which include: The potential interdependence between PHB and PHB2, the pleiotropic nature of PHB, the highly diverse and plastic phenotypes of macrophage *in vitro* and *in vivo*, and the sex dimorphic response of Mito-Ob and mMito-Ob transgenic mouse models.

6.1 The potential interdependence between PHB and PHB2

In the current study, there is a possibility that PHB2 plays a role, independently, compensatory of or synergistically with PHB, in the observed phenotypes in M1 and M2 macrophages. PHB2, a homologue of PHB, shares many similarities with PHB in terms of expression, protein domains, cellular locations, and pleiotropy. Phylogenetic analysis revealed that organisms have at least one version of both PHB and PHB2 equivalents, suggesting these proteins to be indispensable. PHB2 exhibits 53% whole protein sequence identity with PHB and is its closest protein homologue in mammals. Predominately in the mitochondria, PHB and PHB2 have been reported to be interdependent. Over the years of study, the most well-characterized structure is the assembly of PHB/PHB2 heterodimer unit to the high molecular weight oligomers in the mitochondria. The PHB/PHB2 heterodimer unit is formed with a high preference relative to homodimers [20]. This preference may have arisen from a unique and strong complementarity of the heterodimer that is not available in the respective homodimers. Intriguingly, the nuclear and cytosolic roles of PHB2 are relatively distinct and independent from PHB, and heterodimerization

or oligomerization of the two proteins has not been reported in these two compartments. Despite their protein sequence homologies and the knowledge that both proteins are found in all tissue and cell types, no systematic evaluation of the formation of heterodimers has been reported. Therefore, when a function is reported for PHB or PHB2, the participation of the other protein remains unknown. Furthermore, the compensatory role of either protein for one other is only speculative. It is possible that their interdependence extends beyond the mitochondria. Studies that modified PHB or PHB2 expression in the functional analysis should aim to examine the other protein at the same time unless there is an inherent difficulty in parsing the function of the individual protein from that of heterodimers.

6.2 Pleotropic Nature of PHB

Different terms have been used to describe proteins like PHB that possess more than one function, including pleiotropy, multidomain proteins, promiscuity and moonlighting [21]. Measurement of pleiotropic proteins is far from straightforward. Wagner and Zhang proposed two broad types of pleiotropy: multiple molecular functions of a gene (type I pleiotropy) and multiple morphological and physiological consequences of a single molecular function (type II pleiotropy) [22]. PHB can fit into both categories. Type I pleiotropy may be a result of multiple functional domains in the same protein or alternative splicing variants from the same gene locus [22]. The N-terminal hydrophobic domain allows PHB to be anchored to the endomembrane system and perform compartment-specific functions. Cellular fractionation data support PHB localization in multiple organelles and its ability to translocate. The PHB domain is shared by a number of proteins. Its exact function is unknown, but members carrying this domain support membrane microdomain formation and develop putative scaffolds [23,24]. They also share the propensity to

oligomerize, undergo posttranslational modifications and localize to multiple subcellular compartments without a clear targeting sequence [23]. As a key signalling molecule at the crossroad of diverse cell pathways, PHB also possesses type II pleiotropy and can produce diverse physiological consequences. Genotype-phenotype mapping suggests that pleiotropy is predominately type II [22]. For example, PHB plays a dual role in macrophage polarization. Although closely aligned with each other, M1 and M2 polarizations are two distinct cellular mechanisms. This is exemplified by the unique machinery activated and the functional outcome produced. The multiple functionalities of PHB are likely acquired during evolution, as a comparative genome analysis in Arabidopsis suggests that PHB has undergone gene duplication and derived new functions due to evolutionary pressure [25]. These functions may be regulated by proteolysis, where one form is readily degraded, while the other is resistant through oligomerization [26]. The pleiotropic property of PHB is further developed by subcellular localization and post-translational modifications.

6.3 Experimental Model of Macrophages

For investigations in this thesis, I conducted experiments on the murine cell line RAW 264.7 and the splenocytes from Mito-Ob mouse models in the CD-1 mouse background. RAW 264.7 is of the monocytic lineage and was established from Abelson murine leukemia virus-induced tumour in a BALB/c male mouse. This cell line remains robust before passage 30 [27]. A comparison of primary (Bone marrow-derived macrophages) and transformed (RAW 264.7, J774A.1 and IC-21) macrophages of mice origins has found key differences under identical culture conditions, including proliferative ability, cytokine production and surface marker expression [28]. It was also found that RAW 264.7 has generally proinflammatory properties associated with a loss

of differentiated functions, leading to reduced functional plasticity [28]. CD-1 mice are albino outbred mice with great genetic diversity, which mimic the human population [29]. Compared to the inbred BALB/c strain commonly used in immunology, the outbred CD-1 strain offers the opportunity to analyze a broad range of genetic polymorphisms. On the other hand, individual differences can lead to some variations in experimental results. Macrophages from the spleen of male Mito-Ob and mMito-Ob mouse models were used for immunophenotyping. Spleen is the second largest immunological site after the bone marrow. Under the resting condition, splenic macrophages are more M1-like with upregulated expression of TNFα and IL-1β [30]. In contrast, bone marrow-derived macrophages exhibit the most homogeneity and were partially polarized to the M2 phenotype [30]. The propensity to be polarized under the resting state can skew data interpretation and make it difficult to draw conclusions. To elucidate the immune profiles of male Mito-Ob and mMito-Ob mice, adipose tissue macrophages would have been the best cell type to analyze. Though, it's still unclear whether macrophages are monocyte-derive, arise from tissue-specific macrophages or a combination of both. Nonetheless, the use of phenotypically relevant cells is not without technical difficulties, such as the scarce availability and difficult logistics of primary macrophage cells preventing a larger scale use of the cells.

Information about the regulation of immune response in treated animals may not be translational to humans due to the differences between mouse and human macrophages. Namely, these key differences exist in gene expression and the magnitude of polarization. Murine macrophages stably express the surface antigens F4/80, CD11b (Integrin alpha M), CD68 and colony stimulating factor 1 receptor (CSF1R), whereas human macrophages express all but F4/80 [31]. While F4/80 is a defining membrane protein of the murine macrophage population, the human ortholog codes for an eosinophil-specific receptor known as the epidermal growth factor module

containing mucin-like hormone receptor 1 (EMR1) [32]. Interspecies differences in response to LPS were moderately diverse with around 24% of total genes being differentially regulated [33]. One controversial difference is the production of NO [34]. Murine macrophages consistently express iNOS and produce NO in response to LPS and IFNγ. In human macrophages and even other rodent models, iNOS activity was not consistently induced by similar stimuli [34,35]. A single-cell transcriptomics study of human and mouse myeloid cell populations in tumours also revealed macrophage heterogeneity and stressed the need to identify better translational mouse models against human conditions [36].

In this thesis, the M1/M2 categorization of macrophages was used. However, the two states do not fully recapitulate the complex and changing environment macrophages respond to. For example, during malignancy, the distinct population of tumour-associated macrophages is observed to enhance the survival, growth, and metastasis of tumour cells [37]. Recently, a group of metabolically-activated macrophages (MMe) have been characterized through stimulation with glucose, insulin, and palmitate, which mimic the condition of the metabolic syndrome [38]. The latest research proposed a multi-dimensional model of macrophages that incorporates the complex milieu that macrophages encounter *in vivo* [39], which better recapitulates the microenvironment macrophages encounters in the human body. Indeed, macrophages are extremely heterogeneous even within the same tissue and disease state. Therefore, results and analyses from this thesis are limited by the significant heterogeneity in macrophages observed both spatially and temporally within an organism.

6.4 Sex differences

Sex difference is known to exist in immune functions in the body, and sex steroid hormones are integral to these differences. Estrogen, in particular, plays an important role in modulating immune functions. The relationship between immunometabolism and sex differences in immune functions, especially the immunomodulatory role of estrogen, remains largely unknown. Mito-Ob and mutant-Mito-Ob transgenic mouse models helped uncover PHB's important role in the sex differences that inherently exist in adipose and immune functions. Mito-Ob and mMito-Ob mice share metabolic phenotypes, which include sex-neutral obesity but male sex-specific metabolic dysregulation [40]. These findings pointed to the possibility of a protective role of estrogen in immune dysregulation in Mito-Ob and mMito-Ob mice. In preliminary studies, I observed that ovariectomized mMito-Ob mice exhibit significantly reduced cytokine levels in comparison with the control [data not shown]. However, the ovariectomized Mito-Ob mice displayed the opposite. Moreover, manipulating PHB level, or its function in macrophages *in vitro*, affects its response to 17β-estradiol. PHB and PHB2 are known to have a complex relationship with sex steroids [41]. Both have been reported to function as repressors of sex steroid actions and have also been identified as target genes for sex steroids [42,43]. Estrogen has a protective effect on the development of obesity-related lymph node tumours in the female mMito-Ob mice, as ovariectomized mice develop these tumours similar to their male counterparts [14]. Analysis of cytokines in serum samples revealed that female mMito-Ob mice had lower pro-inflammatory cytokines compared with female Mito-Ob mice [data not shown]. Ovariectomy in mMito-Ob mice resulted in further reduction ($P < 0.01$-0.0001). The magnitude of ovariectomy-induced decrease varied widely among different cytokines. Interactions between PHB and sex steroids may include plasma membrane-associated cell

signalling and/or mitochondrial action. Therefore, the relationship between PHB and estradiol-estrogen receptor action requires further investigation.

6.5 References

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CHAPTER 7: CONCLUSIONS, IMPLICATIONS AND FUTURE DIRECTIONS

In 2002, immunometabolism was introduced after the discovery of the link between CD28 activation and glycolysis in T cells [1]. This field aims to understand the impact of immune cells on metabolism and the metabolic needs of immune cells during physiological or pathological processes. PHB is an essential pleiotropic protein that presents a unique opportunity to explore the complex intracellular and intercellular play in immunometabolism. It is one of the many pleiotropic proteins in the body that governs multiple aspects of cell and tissue functioning. The work presented in the thesis contributed to the field of macrophage immunometabolism and enhanced our understanding of PHB function in the important innate immune cell type. Macrophages are known to play multiple roles, from maintaining tissue homeostasis to initiating an innate immune response against microbial invasion. Macrophages are also indispensable in the initiation, progression, and resolution of inflammation, which are highly regulated. In response to inflammation, intracellular signalling pathways such as the STATs, NFκB and MAPKs are activated to regulate the magnitude and duration of this response. Signalling molecules in these pathways are particularly important targets for anti-inflammatory intervention. In this context, PHB emerges as a potential candidate that modulates all these pathways. PHB plays a role in the signalling pathways following macrophage polarization to both M1 and M2 phenotypes and has a global effect on cytokine production. These roles are mediated in part through PHB phosphorylation at Tyr-114, as mutation at this site disrupts IL-4 signalling and mitochondrial respiration in M2. Furthermore, Tyr-114 phosphorylation of PHB is implicated in the PI3K/Akt and MEK/ERK signalling pathways in macrophages, both of which are essential in producing M1- and M2-specific profiles. In addition to phosphorylation, PHB also undergoes palmitoylation, glycosylation, ubiquitination, and nitrosylation; the possible role of these modifications in

macrophage biology has yet to be investigated. The cellular machinery and metabolic profile of activated macrophages vary significantly as a function of the environment. Activated macrophages initially exhibit a rapid pro-inflammatory response, and then obtain the M2 phenotype to resolve inflammation and repair the damaged tissue. This temporal change in phenotype during acute inflammation points to a phenotypic switch through metabolic regulation. PHB was found to differentially alter cytokine productions in M1 and M2 macrophages at the transcriptional level, providing additional evidence for PHB's role as the metabolic switch. In summary, the thesis lays the foundation for PHB regulation of macrophage function and provides an important framework for understanding how cellular pathways may be regulated in multiple functional states of the same cell type. It's worthwhile to investigate PHB function in other immune cells and immune responses, such as T cells and B cells and their activation during adaptive immunity. Identification of direct interacting partners of PHB in the JAK/STAT and NFκB signalling pathways in macrophages could provide a more accurate location of PHB in the signalling cascade.

The findings from the thesis introduce PHB to the field of immunometabolism. Results from macrophages provide a good foundation for the understanding of PHB in a cell-specific and compartment-specific manner. Research going forward would benefit from expanding in areas shown in Figure 7.1.

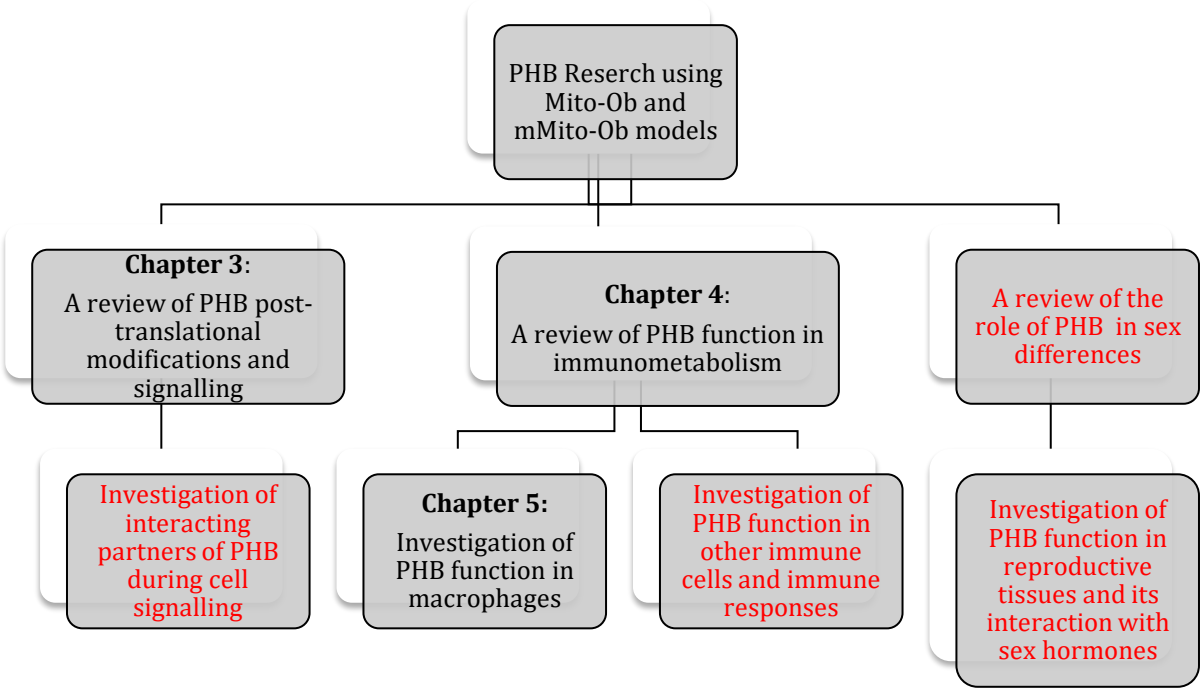

Figure 7.1. Future directions of PHB research based on current knowledge. Black: Topics investigated in this thesis as presented in Figure 2.1. Red: Areas of interest in future research that can be built upon this thesis.

A list of publications since the start of the Ph.D. program.

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