The impact of Tafazzin deficiency on the functional and immunological characteristics of B lymphocytes

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Abstract

Barth syndrome (BTHS) is a rare X-linked genetic disease classically characterized by cardiomyopathy, skeletal myopathy, and neutropenia. It is caused by mutations in the TAFAZZIN gene, which codes for the protein tafazzin (Taz), and this results in alterations in the level and molecular composition of the mitochondrial phospholipid cardiolipin with subsequent disruption in mitochondrial bioenergetics. Impaired mitochondrial function in BTHS patients contribute to increased risk of serious infections. B lymphocytes play a crucial role in the immune response and are responsible for humoral immunity through the production of antibodies. Intact mitochondrial function is essential to support B cell activation, function, and differentiation. Mesenchymal stem cells (MSCs) are immunoregulatory cells which can inhibit the activation of immune cells and they can suppress the immune response. Genetic modifications of MSCs have been shown to modulate their immunosuppressive properties. The aims of my project were to study the effect of Taz deficiency on B lymphocyte activation in both a human and a mouse model of BTHS and study the impact of Taz deficiency on the immunosuppressive function of MSCs and how this modulates B lymphocyte activity and differentiation. We observed that Epstein-Barr virus transformed control and BTHS lymphoblasts failed to enhance surface marker expression in response to lipopolysaccharide (LPS) stimulation and exhibited varied surface marker expression in response to cytosine phosphate guanine-deoxyribonucleic acid (CpG DNA) stimulation indicating that they are a poor model to study BTHS B cell immune function. However, Taz deficiency in B cells isolated from 3-month-old Taz knockdown (TazKD) mice exhibited a decreased ability to be activated by LPS or anti-cluster of differentiation 40 + interleukin-4. In addition, TazKD B cells exhibited reduced glycolysis and oxidative phosphorylation and this correlated with a reduction in their proliferation and immune function upon activation. We observed that MSCs isolated from TazKD mice exhibited an increase in glycolysis and this correlated with an enhanced

proliferation and immunosuppressive properties. Finally, we showed that Taz deficient MSCs reduced proliferation and differentiation of LPS activated B cells. Our results highlight a novel role for Taz in the regulation of the immune system.

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Contribution of Authors

This thesis work is conducted in a sandwich format including multi-author manuscripts. The following section describes the list of author contributions in each manuscript.

Manuscript 1: Barth syndrome: Cardiolipin, cellular pathophysiology, management, and novel therapeutic targets. Published in Molecular and Cellular Biochemistry.

1- **Hana Zegallai:** Prepared and drafted the initial manuscript.
2- **Grant M. Hatch:** Supervised and revised the manuscript.

All authors have edited and approved the final version of the manuscript

Manuscript 2: Impaired surface marker expression in stimulated Epstein-Barr virus transformed lymphoblasts from Barth Syndrome patients. Published in Scientific Reports.

1- **Hana M. Zegallai:** Conceptualized the study, performed and carried out the experiments, acquired and interpreted the data, performed statistical analysis, drafted the manuscript.
2- **Grant M. Hatch:** conceptualized the study, interpreted the data, and revised the manuscript.

All authors edited and approved the final version of the manuscript.

Manuscript 3: Tafazzin deficiency impairs mitochondrial metabolism and function of lipopolysaccharide activated B lymphocytes in mice. Published in The FASEB journal.

1- **Hana M. Zegallai:** conceptualized the study, performed and carried out the majority of the experiments, acquired and interpreted the data, performed statistical analysis, designed figures, drafted the manuscript.
2- **Ejlal Abu-El-Rub:** performed the experiments, analyzed and interpreted the data, designed figures, drafted the manuscript.

3- **Laura K. Cole:** established and maintained the Taz knockdown transgenic mouse model, provided the spleen, performed the experiments.
4- **Jared Field:** performed the flow cytometry for B cell cycle and analyzed the data.
5- **Edgard M. Mejia:** performed and carried out the experiments.
6- **Joseph W. Gordon:** provided reagents and analytic tools.
7- **Aaron J. Marshall:** conceptualized the study and interpreted the data.
8- **Grant M. Hatch:** conceptualized the study, interpreted the data, and revised the manuscript.

All authors edited and approved the final version of the manuscript.

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4- **Genevieve C. Sparagna:** performed the Lipid Mass Spectrometry and analyzed the data.
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1- **Hana M. Zegallai:** conceptualized the study, performed and carried out the experiments, acquired and interpreted the data, performed statistical analysis, designed figures, drafted the manuscript.
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5- **Genevieve C. Sparagna:** performed the Lipid Mass Spectrometry and analyzed the data.
6- **Aaron J. Marshall:** performed, designed, and analyzed the flow cytometry experiments.
7- **Grant M. Hatch:** conceptualized the study, analyzed and interpreted the data, and revised the manuscript.

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List of Abbreviations

3-MGA: 3-methylglutaconic aciduria

A/A: Antibiotic-Antimycotic

Akt: Protein kinase B

ALCAT1: Acyl-CoenzymeA: lysocardiolipin acyltransferase1

ANOVA: Analysis of Variance

APCs: Antigen-presenting cells

ATP: Adenosine Triphosphate

BCR: B cell receptors

Bregs: Regulatory B cells

BTHS: Barth syndrome

Cat.#: Catalogue Number

CD: Cluster of Differentiation

CDP-DG: Cytidine diphosphate-1,2-diacyl-*sn*-glycerol

CI: Complex 1

CII: Complex 2

CII: Complex 3

CIV: Complex 4

CL: Cardiolipin

CLRs: C-type Lectin receptors

CpG DNA: Cytosine phosphate guanine-deoxyribonucleic acid

CSR: Class-switching recombination

CTLA-4: Cytotoxic T lymphocyte antigen 4

CXCR4: C-X-C chemokine receptor type 4

DAMPs: Danger Associated Molecular Patterns

DCs: Dendritic cells

DHA: Docosahexaenoic acid

Dox: Doxycycline

ECAR: Extracellular acidification rate

ELISA: Enzyme-linked immunosorbent assay

ETC: Electron transport chain

EVs: Extracellular vesicles

FAO: Fatty acid oxidation

FBS: Fetal Bovine Serum

FC: Fold change

FO: Follicular

G-CSF: Granulocyte colony stimulating factor

Glut1: Glucose transporter

GSK3: Glycogen synthase kinase 3

H chain: Heavy chain

HGF: Hepatocyte growth factor

h: Hours

IDO: Indoleamine 2,3-dioxygenase

IFNγ: Interferon gamma

Ig: Immunoglobulin

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IL: Interleukin

IMM: Inner mitochondrial membrane

IRFs: Interferon regulatory transcription factors

KC: **Keratinocytes-derived chemokine**

L4-CL: Tetralinoleoyl Cardiolipin

L chain: Light chain

LLPCs: long-lived plasma cells

LPS: Lipopolysaccharide

MAP1LC3: microtubule-associated protein 1 light-chain 3

MAVS: mitochondrial antiviral signaling

MCP-1: monocyte chemoattractant protein-1

µg: Microgram

mg: Milligram

MHC-I: Major Histocompatibility Complex Class I

MHC-II: Major Histocompatibility Complex Class II

MICOS: Mitochondrial contact site and cristae organizing system

mins: Minutes

MIP-2: Macrophage-inflammatory protein-2

µL: Microlitre

MLCL: Monolysocardiolipin

MLCLAT1/HADHA: MLCL acyltransferase-1/trifunctional protein alpha subunit

MSCs: Mesenchymal stem cells

mtROS: Mitochondria ROS

MZ: Marginal zone

NET: Neutrophil extracellular trap

NFAT: Nuclear factor of activated T cells

NK: Natural killer cell

NLRs: NOD-Like Receptors

NOD: Nucleotide-binding oligomerization domain

OCR: Oxygen consumption rate

OMM: Outer mitochondrial membrane

OXPHOS: Oxidative phosphorylation

pAKt: Phosphorylated protein kinase B

PAMPs: Pathogen-associated molecular patterns

PBS: Phosphate buffered saline

PD-L1: Programmed death ligand-1

PG: Phosphatidylglycerol

PGE2: Prostaglandin E2

PGP: Phosphatidylglycerol phosphate

PI: Propidium Iodide

RIG-I like: Retinoic acid-inducible gene-I

PI3K: Phosphatidylinositol-3-kinase

ROS: Reactive oxygen species

PRRs: Pattern-Recognition Receptors

SDF-1: Stromal cell-derived factor 1

SHM: Somatic hypermutations

shRNA: Short Hairpin Ribonucleic Acid

SLE: Systemic lupus erythematosus

SLPCs: short-lived plasma cells

Taz: Tafazzin

TazKD: Taz knockdown

TCA: tricarboxylic acid

TCR: T cell receptors

TD: T-cell dependent

TGF-β: transforming growth factor-β

TH: T-helper

TI: T-cell independent

TLRs: Toll-Like Receptors

TMT: tandem mass tag

TNFα: Tumor Necrosis Factor–alpha

Treg: regulatory T cells

VEGF: vascular endothelial growth factor

WT: Wild type

CHAPTER I. Introduction

Some introduction sections have been published in the Molecular and Cellular Biochemistry Journal as a review entitled **"Barth syndrome: Cardiolipin, cellular pathophysiology, management, and novel therapeutic targets"**.

Hana Zegallai and Grant M. Hatch

Introduction

Barth syndrome (MIM302060) (abbrev. BTHS) is a rare X-linked genetic recessive disorder first described by Dr. Peter Barth in 1983 [1]. BTHS is mainly manifested in infancy and early childhood, and it is characterized primarily by cardiomyopathy, skeletal myopathy, growth retardation, neutropenia, and 3-methylglutaconic aciduria (3-MGA) [2, 3]. The major cause of death in patients with BTHS is heart failure. On the other hand, neutropenia (cyclic, chronic, or intermittent) is the second leading cause of death in BTHS patients due to a high risk of infection [4]. The cardiovascular disease component of BTHS requires careful assessment and management. However, infections associated with the disease require urgent intervention.

BTHS is caused by mutations in the TAFAZZIN gene localized to chromosome Xq28.12 [1–5]. Over 100 different mutations in the TAFAZZIN gene have been described. The TAFAZZIN gene encodes the protein tafazzin (Taz) which serves as a transacylase enzyme involved in the remodeling and maturation of the mitochondrial phospholipid cardiolipin (CL). Taz transfers fatty acyl groups from phospholipids such as phosphatidylcholine and phosphatidylethanolamine to monolysocardiolipin (MLCL) to produce CL [6]. Thus, TAFAZZIN mutations result in CL remodeling impairments which lead to reductions in CL, mitochondrial dysfunction and the development of BTHS [7].

1.1 Cardiolipin structure and biosynthesis

CL (1,3-bis(sn-3'-phosphatidyl)-sn-glycerol) is a specific phospholipid of mitochondrial membranes. It is an anionic phospholipid and is comprised of two phosphatidic acid molecules linked by a glycerol moiety [8]. CL was isolated for the first time from bovine heart by Pangborn [9]. It exhibits a conical structure with a small polar head group and a large hydrophobic tail. CL is an essential phospholipid of the inner mitochondrial membrane where it comprises approximately 15-20% of the inner mitochondrial membrane phospholipid mass

[10]. Unlike other phospholipids, CL is predominantly localized to the inner mitochondrial membrane (IMM) and exhibits a unique dimeric molecular structure: three glycerol groups, two phosphate moieties, and four acyl chains [11]. This unique structure of CL introduces tension into mitochondrial membranes and it is believed that this characteristic structure of CL plays an important role in its functional role in mitochondrial membranes. Moreover the negative charges of CL facilitate its interaction with the outer mitochondrial membrane (OMM) and IMM proteins and this explains why large quantities of CL are found in cristae membranes [12, 13]. In functioning mitochondria, the cristae membranes exhibit a charge. During oxidative phosphorylation, the electrons are transferred through the electron transport chain (ETC) and protons are pumped out of the mitochondrial matrix. The pumping of protons out of the matrix create an electrochemical gradient across the membrane which results in a negative charge on the inner side of the cristae membrane [14].

The molecular species of CL differ between tissues. For example, mammalian cardiac mitochondrial CL consists of approximately 80-90% of linoleic acid (C18:2), the most abundant CL species being tetralinoleoyl-CL (L_4CL). The tissue specific acyl chain composition of CL is important for proper mitochondrial function [15]. For example, enrichment in L_4CL is critical for oxidative phosphorylation and ETC function in the heart. Decreasing L_4CL in the CL cardiac profile resulted in mitochondrial dysfunction [15, 16]. Previous studies showed that disruption of the specific acyl chain composition of CL leads to a decrease in CL mass and plays a major role in cardiac pathophysiology [17, 18]. Since L_4CL is essential for optimal mitochondrial function in the heart, replacing linoleic acid with other fatty acids leads to decrease in the activity of the proteins in IMM due to changes in membrane characteristics. For example, high levels of docosahexaenoic acid (DHA) (22:6) in CL acyl chain composition results in disturbed protein-lipid interactions due to increase in the fluidity of the mitochondrial membranes [19].

De novo biosynthesis of nascent mammalian CL begins with formation of cytidine-5'-diphosphate-1,2-diacyl-*sn*-glycerol (CDP-DG) from a reaction between phosphatidic acid and cytidine triphosphate and is catalyzed by mitochondrial assembly and maintenance protein Tam41 [20, 21], otherwise known as CDP-DG synthetase. In the next step, phosphatidylglycerol phosphate (PGP) is synthesized by transferring a phosphatidyl group from CDP-DG to the sn-1 position of glycerol-3-phosphate and this is catalyzed by phosphatidylglycerol phosphate (PGP) synthase. PGP is then rapidly dephosphorylated by protein tyrosine phosphatase mitochondrion-1 to form phosphatidylglycerol (PG). The last step in *de novo* biosynthesis of nascent CL is the condensation of another molecule of CDP-DG with PG catalyzed by CL synthase [22, 23].

After biosynthesis of nascent CL, nascent CL undergoes remodeling by deacylation and subsequent resynthesis. Nascent CL is deacylated to MLCL by phospholipase A_2. Mature CL is then resynthesized (remodeled) by either Taz, MLCL acyltransferase-1/trifunctional protein alpha subunit (MLCLAT1/HADHA) or acyl-CoenzymeA:lysocardiolipin acyltransferase1 (ALCAT1). Taz and MLCLAT1/HADHA resynthesize CL in mitochondria. ALCAT1 resynthesizes small fractions of MLCL to mature CL in the mitochondria associated membrane (Figure 1.1) [24]. Both MLCLAT1/HADHA and ALCAT1 are acyltransferases which transfer acyl chains from fatty acyl-Coenzyme A to MLCL to form mature CL [6, 25, 26]. In BTHS lymphoblasts a compensatory upregulation of MLCL AT-1 has been reported [27]. Transfection of BTHS lymphoblasts with a MLCL AT-1 construct resulted in elevated CL and enhanced mitochondrial function by reducing mitochondrial ROS, improving basal mitochondrial respiration and proton leak [28]. Unlike MLCL AT-1, ALCAT1 is linked to pathogenic CL remodeling leading to increase oxidative stress and mitochondrial dysfunction [29].

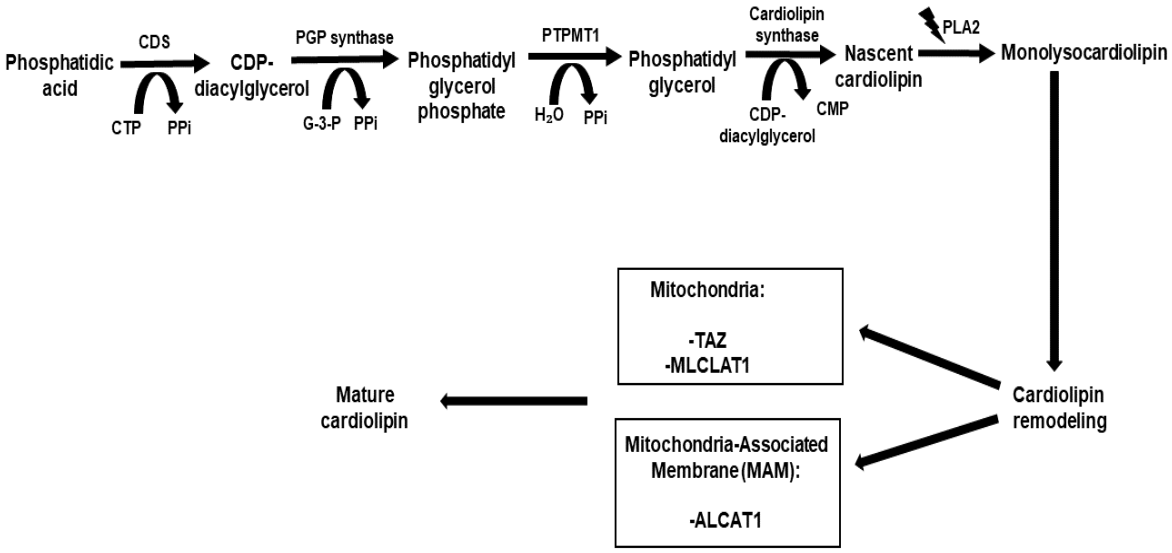

Figure 1.1 *De novo* biosynthesis and remodelling of Cardiolipin. CL biosynthesis is catalyzed by several enzymes to generate nascent CL as follows: CDS: CDP-DAG synthase; CTP: cytidine triphosphate; CDP- diacylglycerol: cytidine diphosphate-diacylglycerol; PGPS: phosphatidylglycerol phosphate synthase; PTPMT1: protein tyrosine phosphatase mitochondrial 1; Cardiolipin synthase. Nascent CL is deacylated to MLCL: monolysocardiolipin by PLA_2: phospholipase A_2. Then, CL is remodelled to mature CL with specific enzymes including TAZ: tafazzin; MLCLAT1: monolysocardiolipin acyltransferase 1, and ALCAT1: acyl-CoA:lysocardiolipin acyltransferase 1.

1.2 Cardiolipin function

CL is essential for mitochondrial structure and function. It plays an important role in mitochondrial cristae organization and biogenesis, mitochondrial membrane architecture, mitochondrial bioenergetics and oxidative phosphorylation, lipid-protein interactions, and mitochondrial supercomplexes assembly [30]. Mitochondrial cristae contain a significant amount of CL and provide a large surface area for mitochondrial bioenergetics reactions such as oxidative phosphorylation for ATP production and mitochondrial respiration activity. In addition, CL is important for mitochondrial fission and fusion and cell apoptosis [24]. CL is essential for proper function of mitochondrial respiratory super complexes composed of complex I (NADH- ubiquinone oxidoreductase), complex III (ubiquinone-cytochrome c oxidoreductase), complex IV (cytochrome c oxidase), and complex V (ATP synthase) [31–

33]. Impairments in CL biosynthesis, remodeling, and/or metabolism may lead to metabolic diseases, including cardiovascular diseases and type 2 diabetes [34, 35], and of course the life-threatening genetic disorder BTHS. Deficiency in CL resulting from Taz gene mutation affects mitochondrial biogenesis and leads to a significant decrease in ATP synthesis. Moreover, mitochondrial morphology and inner mitochondrial membrane organization are disrupted in CL deficiency [36].

CL exhibits two negatively charged phosphates. Therefore, it induces negative membrane curvature. It thus has the ability to trap protons at the inner mitochondrial membrane, and interact with proteins in the outer and inner mitochondrial membrane [37]. CL is required for the ADP/ATP carrier activity and 6 molecules of CL are tightly bound to it [38]. The absence of CL synthase, with a resulting loss in CL, was shown to affect mitochondrial function by disrupting mitochondrial membrane potential and result in impairments in ADP/ATP carrier activity on the inner mitochondrial membrane [39]. The inner mitochondrial membrane proton gradient is disrupted by decreased CL content, and this affects the inner membrane microenvironment and reduces protein carrier and respiratory enzymes activity in the inner mitochondrial membrane leading to membrane proton gradient disruption [39, 40]. The unique conical shape of CL and its negative charge allow CL to interact with different proteins such as prohibitins, stomatin like-2 protein, mitochondrial contact site and cristae organizing system (MICOS), and microtubule-associated protein 1 light-chain 3 (MAP1LC3) [41, 42]. Prohibitins are proteins found in inner mitochondrial membrane involved in phospholipid homeostasis and cellular signaling. CL molecular composition was shown to be altered in HEK293 cells by knockdown of prohibitin 2 [41, 43]. MICOS is a large heterogeneous protein that is present in cristae junctions and plays an important role in cristae formation. MAP1LC3 is a protein that interacts with CL and cellular membranes. MAP1LC3 acts as a marker for autophagosome recruitment [41]. As indicated

above, CL plays an essential role in apoptosis. In the intrinsic apoptotic pathway (mitochondrial- mediated apoptosis), CL interacts with caspase-8 and recruits caspase-8 to the outer mitochondrial membrane [44]. CL also is required for the recruitment of intrinsic apoptotic factors such as tBid from cytosol to the inner mitochondrial membrane [45]. In addition, CL plays an important role in mitophagy, a selective mechanism to eliminate damaged mitochondria by autophagy, through interaction with other proteins such as MAP1LC3 [42].

CL is critical for mitochondrial supercomplexes stability, optimum function and structure, and CL deficiency can lead to dissociation of supercomplexes and a decrease in energy production [46]. CL thus acts as the glue that holds mitochondrial respiratory supercomplexes together [47]. BTHS models show dissociation of supercomplexes. In addition, reduction in supercomplexes formation and activity is observed in many tissues of Taz knockdown (TazKD) mice, an animal model of BTHS, compared to age matched wild type mice [48]. Mitochondrial reactive oxygen species (ROS) production increases when supercomplexes are disrupted and this leads to CL peroxidation. The CL molecular structure is very sensitive to lipid peroxidation and CL peroxidation initiates apoptosis through the release of cytochrome c [49]. In addition, since CL is localized to the inner mitochondrial membrane where generation of ROS occurs, this itself makes CL susceptible to peroxidation [24]. An imbalance between mitochondrial ROS and antioxidant enzymes such as superoxide dismutase may lead to a significant increase in cellular damage [50].

1.3 Main clinical phenotypes in BTHS

1.3.1 Cardiac effects

Cardiovascular disease is the most significant problem in BTHS patients. There is a wide variability of cardiac phenotypes of BTHS, including dilated cardiomyopathy, left ventricular

non-compaction, endocardial fibroelastosis, ventricular arrhythmias, and sudden cardiac death [51]. Mitochondria generate most of the energy needed by the cardiac tissue and are the primary source of the energy in cardiomyocytes [52]. The oxidative phosphorylation process is impaired in BTHS patients. Decreasing CL mass in BTHS mitochondria result in instability of ETC complexes, acceleration of ROS formation, and affect mitochondrial biogenesis and dynamics (Figure 1.2) [52]. Mitochondrial dysfunction is the main cause of cardiomyopathy and heart failure in BTHS patients as cardiac metabolism is strongly dependent on oxidative phosphorylation as a source of energy. TAFAZZIN mutations in these patients result in CL deficiency. Since CL is essential for mitochondrial cristae morphology, oxidative phosphorylation process and normal mitochondrial structure and function (as indicated above), CL deficiency has been implicated in the pathophysiology of the heart failure and cardiac arrhythmias in BTHS patients.

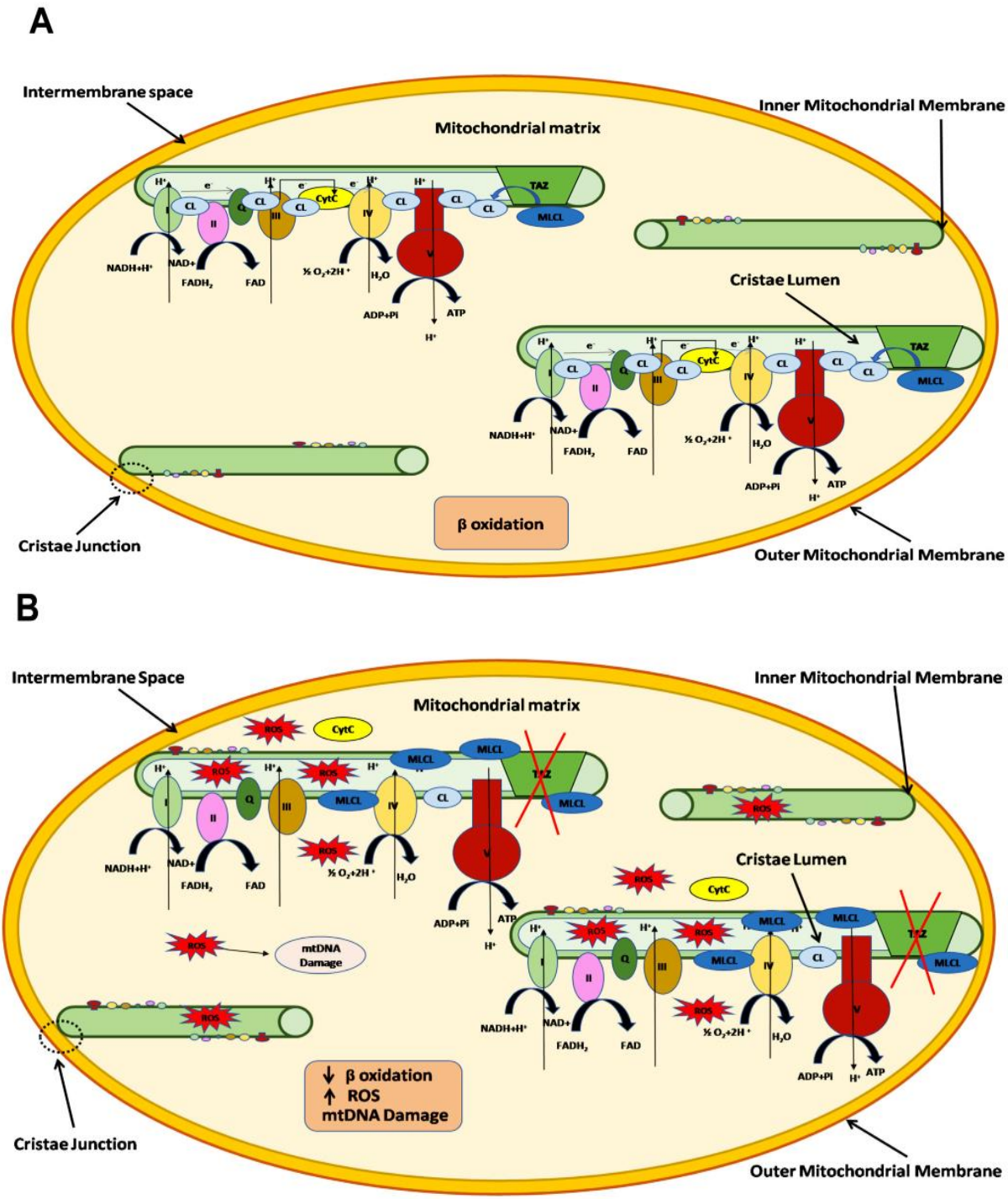

Figure 1.2 Mitochondria in normal and BTHS cells. In normal mitochondria, CL aids in the assembly of the respiratory chain complexes into supercomplex structure to facilitate electron transfer during the oxidative phosphorylation process (**a**). In BTHS mitochondria, with CL deficiency, the respiratory chain complexes are destabilized causing release of cytochrome C (Cty c) and increase reactive oxygen species (ROS) production resulting in impairment of oxidative phosphorylation and decrease in ATP production (**b**).

1.3.2 Neutropenia

Neutropenia is a major haematological abnormality in patients with BTHS and may cause severe infection. As indicated above, although cardiomyopathy is the major symptom of BTHS, some patients do not have cardiomyopathy at diagnosis but exhibit infections from infancy [4]. The molecular mechanisms underlying neutropenia in BTHS are unclear. Although BTHS lymphocytes showed increase in mitochondria size and mass, reduced cristae formation and variable cristae distribution [53], neutrophils in BTHS patients exhibit normal motility, killing activity, phagocytosis, mitochondrial shape and mass [54]. However, neutrophil clearance may be increased by tissue macrophages because of the increase of phosphatidylserine exposure on their surface. Phosphatidylserine is normally an intracellular membrane phospholipid and its exposure on the plasma membrane is a key apoptosis marker. A previous study showed that increasing ROS in BTHS neutrophils induced phosphatidylserine exposure and enhanced neutrophil clearance [53]. Human HL60 myeloid progenitor cells and U937 myeloid cells have been used as *in vitro* models to study the impact of Taz deficiency. Knockdown of the TAFAZZIN in human HL60 cells or U937 cells using an shRNA approach increased the number of annexin V-positive cells, caspase-3 activity and cytochrome c release from mitochondria [55]. In U937 cells these markers of apoptosis were reduced by inhibition of caspase activity using zVAD-fink, a specific caspase inhibitor.

1.3.3 Skeletal muscle metabolic dysfunction

Skeletal muscle atrophy and cardiac dysfunction in patients with BTHS result in impairments in fatty acid oxidation and elevate amino acid utilization [56, 57]. The alteration in myocardial and whole-body substrate metabolism may worsen the pathology of chronic heart failure in these patients. Patients with BTHS have higher proteolytic rate than unaffected males [58]. Alteration in CL remodeling and fatty acid composition may cause alteration in substrate metabolism and this could contribute to the cardiomyopathy and skeletal myopathy

in patients with BTHS [18]. Growth delay and decrease of the oxidative phosphorylation in skeletal muscle during exercise may alter the metabolism of fatty acid and proteins in skeletal muscle. Therefore, other substrates such as glucose and amino acids are used to produce the energy if fatty acid oxidation is not efficient enough to produce energy.

1.4 Immune system

The immune system is comprised of a large network of cells and molecules that mediate protection from diseases especially infectious disease. The immune cells are responsible for producing and coordinating the response against foreign substances which is known as the immune response. The immune response is an essential reaction of the immune system that recognizes and defends the body from microbes and foreign macromolecules such as proteins and polysaccharides [59]. The immune system is composed of two parts: Innate (general) immune system and adaptive (specific) immune system. These two systems are closely connected and work together to provide an adequate immune response.

1.4.1 Innate immunity

The innate immune system (natural immunity) is the first line of defense against antigens in our body. The innate immune system provides rapid and non-specific immune response to different types of antigens. The innate immune system consists of different components including physical and chemical barriers such as epithelial and mucous membranes; phagocytes (including neutrophils, monocytes, and macrophages), natural killer (NK) cells, and dendritic cells (DC); cellular receptors including Toll-like receptors; cellular mediators that regulate the inflammation process and mediate the connection between the innate immune system cells such as cytokines; and serum proteins such as complement system and inflammation mediated proteins [60]. The function of the innate immune system can be divided to three levels 1) Prevention and elimination of the infection, 2) elimination of the pathogens and damaged host cells, and 3) optimization of the immune response through

stimulation of the acquired immune response [61]. The innate immune cells mediate most innate immunity and are responsible for recognition and elimination of foreign microorganisms before they enter physical barriers. Most of these cells are present near the epithelial cells and in the physical barriers such as the skin, mucosal surfaces of respiratory, gastro-intestinal, and genitourinary tracts.

Innate immune cells recognize a diversity of microbes in the cytoplasm and extracellular space and signal through specific receptors called Pattern-Recognition Receptors (PRRs). PRRs can detect pathogen molecules known as pathogen-associated molecular patterns (PAMPs) such as the lipopolysaccharide (LPS) of Gram-negative bacteria. In addition, these receptors respond to signals from damaged tissues of host cells known as Danger Associated Molecular Patterns (DAMPs) [62]. There are several subfamilies of PRRs including Toll-Like Receptors (TLRs) which recognize extracellular and endosomal PAMPs; C-type Lectin receptors (CLRs) which are a large family of PRRs involved in a wide range of biological processes such as cell-cell adhesion and cell–cell interactions; NOD-Like Receptors (Nucleotide-binding Domain Leucine-rich Repeat containing or NLRs) which are cytoplasmic receptors that recognize foreign molecules in the cytosol; and RIG-I like Receptors (retinoic acid-inducible gene-I-like receptors) RLRs which are involved in the recognition of intracellular viral RNA [63]. During the innate immune response, phagocytic cells such as neutrophils and macrophages play an important role in destroying and ingesting the microbes through internalization (phagocytosis) and production of proinflammatory cytokines. Phagocytosis is a crucial cellular process to engulf and eliminate the microorganisms and foreign molecules and is mediated by PRRs on the phagocytes. Neutrophils are considered a front line of host defense in response to bacterial infection and are the most abundant leukocyte cells during the inflammation process. During infection, neutrophils recruit and migrate to injury sites through chemotactic stimuli. In addition to phagocytosis, neutrophils can kill the

bacteria by inducing oxygen-dependent and oxygen-independent microbicidal systems. Oxygen-dependent microbicidal mechanisms are mediated by production of superoxide through the NADPH oxidase system. Reactive oxygen species (ROS), such as superoxide radicals and hydrogen peroxide, are a potent antimicrobial mechanism of neutrophils and are highly effective at killing bacteria. Oxygen-independent mechanisms are mediated by antimicrobial peptides and enzymes that are stored in specific granules in the cytoplasm of neutrophils [60, 64].

Another cell that plays an important role in the innate arm of the immune system is the Natural killer cell (NK). Although NK cells are a type of lymphocyte they have functions associated with the innate immune system. NK cells have cytotoxic activity and provide an immunomodulatory effect during cell-mediated response through recognition and killing of infected cells. NK cells secrete interferon gamma (IFNγ) which is able to directly destroy phagocytized microbes. NK cells are involved in negative recognition through detection of abnormal cells that exhibit low class I major histocompatibility molecules (MHC-I) expression levels. During viral infection the expression of MHC-1 molecules on the surface of infected cells is downregulated. Therefore, these infected cells are detected and destroyed by NK cells [65]. Another type of innate immune cell is the mast cell. Mast cells are involved in the early stage of innate immunity and increase capillary permeability and augment local inflammation. Upon activation, mast cells release inflammatory mediators, cytokines, and proteases to destroy microbes [66]. Although the innate immunity is mainly mediated by cells, soluble molecules such as complement system are present in the blood and extracellular fluids play an important role in the innate immune response. These soluble molecules aid in the clearance of pathogens by binding to them and enhancing phagocyte recruitment to the infection site. The complement system is considered the most important type of these soluble molecules. Activation of the

complement system leads to the production of potent proinflammatory mediators and proteolytic cascades which result in opsonization and lysis of microbes [67].

1.4.2 Adaptive Immunity

The adaptive immune response is divided to two categories: 1. cell-mediated responses which are mediated by T-lymphocytes, and 2. Humoral (antibody)-mediated responses which are mediated by B-lymphocytes.

A- Cell-mediated responses

Cell-mediated immunity or cellular immunity is carried out by T lymphocytes. This type of immune response is responsible for elimination of intracellular microbes that proliferate inside the host cells and cannot be removed by circulating antibodies. During cellular immunity, cytotoxic T-lymphocytes respond to pathogen antigens and eliminate the abnormal and damaged cells, such as virus and bacteria infected cells, through induction of apoptosis. T lymphocytes secrete a variety of cytokines that stimulate other immune cells including phagocytes. In addition, natural killer cells and macrophages are activated to destroy the intracellular pathogens [68].

B- Humoral immune responses

Humoral immunity is carried out by antibodies that are produced by B lymphocytes. During B cell stimulation, B cells differentiate to antibody-secreting plasma cells and secrete antibodies. Antibodies are specialized molecules that bind to and recognize specific microbial antigens. After binding with antigens, antibodies neutralize the infectious microbes and eliminate the antigen through opsonization and phagocytosis [69].

In contrast to the innate immune system, the adaptive immune system can react and remember specific pathogens and respond more vigorously to recurrent infections. Therefore, the adaptive immune system provides long term defense and protection against the same

microbes again by developing a memory response. The adaptive immune system consists of two major components which are T and B lymphocytes [68].

The activation of T and B lymphocytes is considered one of the most complex processes of the immune response. These are the only cells that able to detect and respond to a particular antigenic epitope. The activation of adaptive immunity starts with a specific process known as antigen presentation which is mediated by antigen-presenting cells (APCs) such as dendritic cells, phagocytes, and B cells. The dendritic cells (DCs) are considered the most specialized APCs and are the most abundant immune cells in the body surfaces and tissues [70]. DCs play an important role in the immune response initiation and maintenance and exhibit a unique ability to promote activation and differentiation of naive T lymphocytes. Furthermore, DCs contribute to the immune responses of B lymphocytes and NK cells [71].

An antigen is presented on the cell surface either through a major histocompatibility complex (MHC) class I or an MHC class II. MHC class I molecules are present in all nucleated cells and are expressed on their surfaces. However, MHC class II molecules are constitutively expressed on the surface of professional APCs. Under normal physiological conditions, MHC class I bind with peptides from digested proteins within the cell then transport the peptides to the plasma membrane [72]. When MHC-I are expressed on the surface of cells, it binds to $CD8^+$ T cells through the CD8 co-receptor leading to activation of $CD8^+$ T cells. The activation of $CD8^+$ T cells leads to generation of the immune response against the specific peptide and they secret cytotoxic mediators to kill the cell that present the foreign peptide [73]. MHC-II molecules play a critical role in the adaptive immunity by presenting the antigens from exogenous sources to T lymphocytes. Once an antigen is engulfed by antigen-presenting cells, it is degraded to peptides by proteolytic enzymes in the endosomal vesicles. These microbial peptides bind with MHC-II in the endosome. Following this binding, MHC-II and the peptide

complex is trafficked to the cell surface and recognized by immature CD4+ T helper cells through T cell receptors [72].

1.5 B lymphocytes development

B lymphocytes play a critical role during immune response and are responsible for antibody production. Furthermore, they are regarded as APCs involved in activation, differentiation, and expansion of T lymphocytes. Moreover, B lymphocytes are able to produce a variety of cytokines including interleukins (such as IL-6), pro-inflammatory cytokines (such as Tumor Necrosis Factor–alpha [TNF]α), and anti-inflammatory cytokines (such as IL-10) [74]. Each T and B lymphocyte expresses a specific receptor to recognize a particular antigen. These highly specialized surface receptors known as T cell receptors (TCR) and B cell receptors (BCR), respectively. Unlike BCR, TCR can only recognize antigen peptides that are presented by MHCI and MHCII molecules on APCs. However, the BCR has the capability to recognize antigens before being processed by APCs through binding of the antigen to specific immunoglobulin (Ig) B cell surface receptors [74]. B cells arise from haematopoietic stem cells in the bone marrow and then they migrate to peripheral tissues such as spleen and lymph nodes. B-cell development occurs through several stages via the rearrangement of Ig heavy chain (H chain) and light chain (L chain) genes. The H and L chains comprises the Immunoglobulins molecules that are expressed on the surface of B cells [75]. Pre-pro-B cells are considered the earliest precursors of B cells and are characterized by a lack of BCR elements expression. Pre-pro-B cells differentiate into pro-B cells through rearrangement of the Ig heavy chain genes and expression of pre-BCR. Pro-B cells proliferate and differentiate into pre-B cells through pre-BCR signalling. Pre-B cells rearrange their Ig light chain and differentiate into immature B cells. Immature B cells are considered the first B cells that express a unique and specific functional BCR on their surfaces. Immature B cells mainly express IgM on their surface and they migrate from the bone marrow to the peripheral tissues [76]. In the spleen, IgM^+ immature

B cells differentiate into transitional B cell stages known as T1 and T2 B cells. T1 and T2 B cells are considered precursors of naïve B cells. T1 and T2 B cells have short half-lives and only about 10-30% of T1 and T2 B cells develop to naïve B cells. Another type of transitional B cell is the T3 B cell. Unlike T1 and T2 B cells, T3 B cells do not differentiate to mature B cells. T3 B cells represent an anergic B cell subset that prevents autoreactivity with self-antigens and thus prevent the development of autoimmunity [77, 78].

1.6 B cell types

B lymphocytes can be divided into two main sub-lineages includes B1 cells and B2 cells. B1 cells are long-lived cells that make up the majority of B lymphocytes in newborns, and they are decreased in adults to a small percent. They are mainly found in the peritoneal and pleural cavities. B1 cells are considered as part of innate immune system that provide natural immunity. B1 cells are self-renewing cells that naturally produce antibodies. B2 cells are short-lived cells that constitute the highest percentage of B lymphocytes. B2 cells are metabolically quiescent cells and adaptively interact with an antigen by secreting specific antibodies during activation in secondary lymphoid tissues such as spleen and lymph nodes. Therefore, B2 cells play the main role in the adaptive immunity [79, 80].

B-1 cells can be distinguished from B-2 cells by the following phenotype: $CD19^{hi}$, IgM^{hi}, IgD^{lo}, $CD23^{-}$, $B220^{lo}$, $CD5^{\pm}$ cells. Furthermore, B-1 cells are subdivided into B-1a and B-1b subsets based on CD5 marker expression. B-1a cells are characterized by expression of $CD5^{+}$ on their surfaces. However, B-1b subsets do not express CD5 on their surfaces. Both B1 subsets (B-1a and B-1b) are generated from fetal liver precursors. In addition to natural antibodies production, B-1 cells secrete cytokines such as IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF). B-1a B cells mainly secrete natural IgM antibodies

against self-antigen. However, B-1b cells produce antibodies as a result of T-independent B cell activation mainly against the bacteria and provide long term protection (Figure 1.3) [81].

There are two main populations of immunological B lymphocytes including conventional B lymphocytes and innate-like B lymphocytes. Conventional B lymphocytes are responsible for adaptive immunity and they are characterized by maturation and differentiation to plasmablasts and plasma cells which secrete specific antibodies. Conventional B cells include transitional, germinal center, follicular, and memory B cells. In contrast to conventional B lymphocytes, innate-like B lymphocytes produce the natural antibody spontaneously. The innate-like B lymphocytes phenotype include B-1 cells and marginal zone B cells [79].

Typically, there are three subsets of naïve B cells including B-1 B cells, marginal zone B cells, and follicular B cells. Each B cell subset is unique and has a different function, location, migration ability, and immunological response to T-dependent and T-independent antigens.

The marginal zone (MZ) B cells are self-renewing and non-recirculating cells which reside in the MZ of the spleen. MZ B cells are characterized by expressing specific cell surface markers including $IgM^{hi}IgD^{lo}$ $CD1^{hi}CD21^{hi}CD22^{hi}CD23^{lo}$. MZ B cells play an important role in the early immune response through rapid activation and antibody production. MZ B cells are considered innate-like cells which mainly produce antibodies against bacteria or self-antigens. These natural antibodies play a critical role in prevention of autoimmune diseases. Like B-1 B cells, MZ B cells spontaneously produce natural antibodies and produce antibodies after stimulation through the T independent pathway. MZ B cells mainly mediate the immune response for T independent antigens such as LPS. After activation, activated B cells migrate out of the MZ in the spleen and differentiate to plasma cells. Moreover, MZ B cells may contribute to the immune response for T dependent antigens via direct activation with T-

dependent antigens and interact with helper T cells or through transfer of the pathogens to Follicular (FO) B cells [82, 83].

FO B cells are naïve, mature, recirculatory B cells which recirculate in the bone marrow and the spleen. FO B cells migrate through the blood and the lymph to B lymphocytes clusters (follicles) in the spleen and lymph nodes. FO B cells are considered the main B cells that mediate T-dependent humoral immunity. FO B cells have a highly diverse immunoglobulin repertoire and plays a crucial role in producing antigen-specific antibodies. Activation of FO B cells leads to activation of T cells through CD40 signaling. FO B cells interaction with T cells results in isotype class switched antibodies and production of high affinity antibodies. FO B cells can differentiate into long-lived plasma cells after activation through germinal centre reactions. However, if FO B cells fail to exhibit germinal centre reactions after activation they differentiate into short-lived plasma cells. These short-lived plasma cells secrete low-affinity IgM for 2 or 3 days to initiate the immediate response to infection before undergoing apoptosis [83].

During activation of FO B cells with T cell-dependent antigens, the germinal centers are transiently formed within secondary lymphoid tissues such as the spleen and lymph nodes. Germinal centers are a microenvironment structure required for the humoral immunity response. In germinal centers, B cells undergo proliferation, differentiation, and secretion of higher affinity antibodies through Ig class switching, and formation of long-living plasma cells and memory cells. Long-lived plasma cells mainly reside in the bone marrow but may also be concentrated in the spleen. These cells are able to secrete high-affinity antibodies for a long period of time (months) without re-exposure to antigen or cell division and provide long term protection [84].

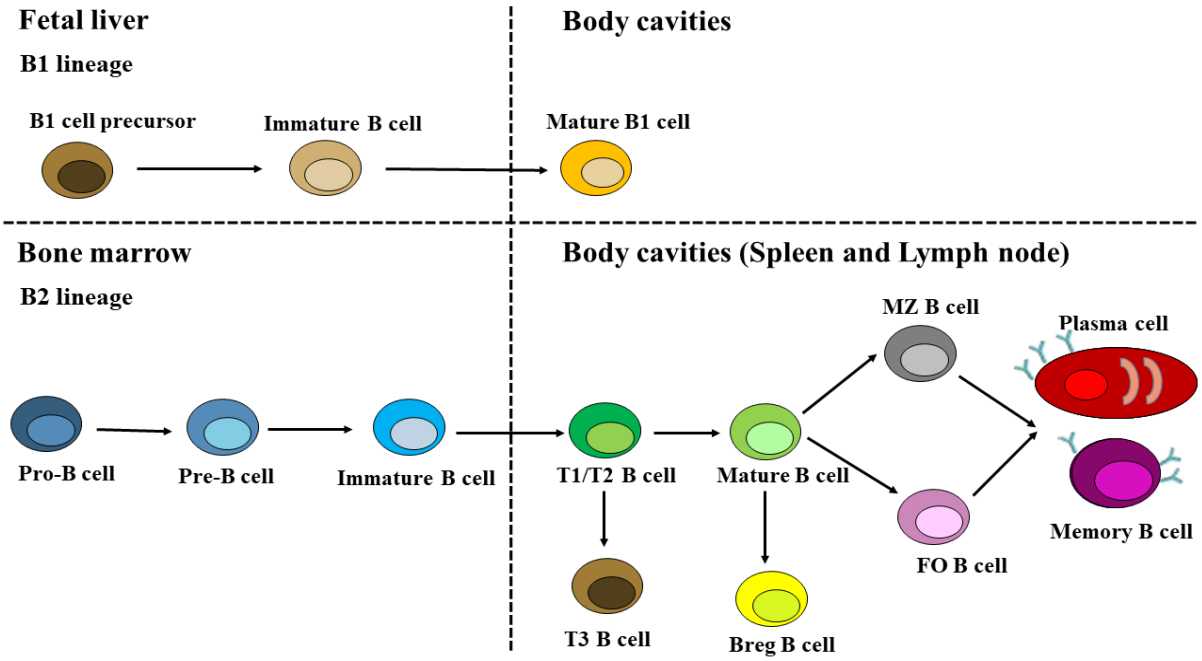

Figure 1.3 Representative illustration for B cells subsets and development. Representative illustration for B cells subsets and development. B lymphocytes can be divided into two main sub-lineages including B1 and B2 lineages. B1subsets are generated from fetal liver precursors and migrate to body cavities as mature B1 cells. B2 lineage undergoes a series of differentiation stages in the bone marrow before migrating to the spleen and lymph nodes including pro-B and pre-B cells. Then, the immature B cells migrate to secondary lymphoid tissues (i.e., lymph nodes and spleen) from bone marrow and differentiate into T1/T2 B cells and T3 B cells. After activation of mature B cells, mature B cells differentiate into marginal zone B cells and/or follicular B cells which subsequently differentiate into plasma cells and memory B cells. In addition, mature B cells can be differentiated to regulatory B cells.

1.7 B lymphocyte activation

B lymphocytes can be activated through T-cell independent (TI) or T-cell dependent (TD) antigens. TI antigens can stimulate B cells without requiring additional help from T cells. TI antigens are large polymers such as lipopolysaccharide and polysaccharide which contain repetitive epitopes in their structure which allow the cross-linking between multiple BCRs to initiate the activation. Due to the absence of T cell help, a second signal needs to be derived from another source such as toll-like receptors which are also essential for B cell activation [85]. Unlike TI antigens, TD antigens require assistance from helper T cells to stimulate B cells. During B cells activation, the interaction between B and T cells occurs through the CD40

ligand, which presents on activated helper T cells surface, and as CD40 protein expressed on the surface of B cells. In addition to this cell-to-cell interaction, the helper T cells produce cytokines such as IL-4 and IL-2 that increase B cells stimulation [86].

1.8 Mitochondria function in immune cells

Immune cells are considered as highly mobile and functional cells which are continuously exposed to a variety of immunological environments with limited source of nutrients. Therefore, immune cells need to reprogram metabolic pathways according to activation state. Mitochondria play a crucial role in cellular bioenergetics in all cells including immune cells. The ATP that is produced through oxidative phosphorylation in mitochondria is required to generate and regulate the immune response during immune cell activation, especially the adaptive immune response [87]. Furthermore, the production of ROS from mitochondria (mtROS) plays an important role in immune system cells and act as signaling molecules and can affect activation and interaction of immune cells, host defense mechanism, and suppression of the immune system [88]. mtROS are mainly produced from complex I and III in the mitochondrial electron transport chain. mtROS promotes inflammatory cytokine secretion and can directly kill pathogens through its antimicrobial activity [89]. In addition, mtROS are important for lymphocyte activation. Activation of T cells through T cell receptors leads to an increase in mtROS generation and rapid T cells proliferation and differentiation. It was reported that mtROS enhance transcription factors and activation of nuclear factor of activated T cells (NFAT) and increased IL-2 and IL-4 expression [90]. Inhibition of mtROS by mitochondrial antioxidants, such as mitovitamin E, decreased the activation of $CD4^{+}$T cell and reduced IL-2 secretion [91]. In addition, mtROS acts as a signaling molecule during activation of B cells and antibody production via heme synthesis. mtROS can suppress differentiation of antibody-secreting plasma cells and enhance class-switch recombination by inhibiting heme synthesis. For example, heme can enhance Blimp-1 transcription, an essential plasma cells

regulator, and increase plasma cells differentiation. On the other hand, heme can bind with Bach2, an important transcription factor for class-switch recombination, *in vitro* and inhibit Bach2 activity [91].

Mitochondria play an important role in NLRP3 Inflammasome activation. The NLRP3 Inflammasome is an intracellular complex protein which plays a critical role in the innate immune system through regulation of pro-inflammatory cytokine secretion such as interleukin-18 (IL-18) and IL-1β. Previous studies showed that mtROS are the main source that activate the NLRP3 Inflammasome [92]. Furthermore, mitochondria can provide antiviral signaling and contribute to the immune response against viruses by initiating the inflammatory response. Mitochondria contain signaling proteins in their outer membrane called mitochondrial antiviral signaling (MAVS) proteins. During viral infection, the viral RNA can activate the MAVS leading to inflammatory gene expression through transcription factor NF-κB and interferon regulatory transcription factors (IRFs) pathways. In addition to viral RNA, mtROS can activate MAVS and promote gene expression. Studies have showed that MAVS can drive NLRP3 oligomerization resulting NLRP3 activation [91].

1.9 Metabolic pathways in neutrophils, B and T lymphocytes

Metabolic pathways are essential for immune cell activation and differentiation. Therefore, metabolic modulation could result in impairment of immune system function and development of chronic diseases. Changes in oxidative phosphorylation, tricarboxylic acid (TCA) cycle, and fatty acid oxidation (FAO) result in alteration of the immune cell response. Most of the innate immune cells such as neutrophils are dependent on glycolysis as their main source of energy. However, mitochondria are closely related to their immune function.

1.9.1 Metabolic pathways in neutrophils

Neutrophils are the most abundant white blood cells in the body and they quickly respond to pathogens during inflammation. Neutrophils have a short life span and are considered as terminal differentiated cells. Therefore, they are mainly dependent on glycolysis rather than oxidative phosphorylation to generate ATP. Glycolysis in neutrophils is the main metabolic pathway required for cellular functions such as ROS production, chemotaxis secretion, and neutrophil extracellular trap (NET) formation. Compared to other immune cells, neutrophils have a lower number of mitochondria and inhibition of oxidative phosphorylation showed no effect on energy production in mature neutrophils [93].

1.9.2 Metabolic pathways in T lymphocytes

T lymphocytes metabolic pathway switch is dependent on their proliferation and effector functions. Resting T cells (naïve T cells) are mainly dependent on oxidative phosphorylation for their cellular function. However, T lymphocytes undergo metabolic reprogramming after stimulation. Activation of T cells by antigen through TCR leads to a shift in their metabolic pathway from OXPHOS to glycolysis. Following naïve T cells activation, they rapidly proliferate and differentiate into effector cells that produce cytokines such as TNFα and IFN-γ. These activated T cells are largely dependent on glycolysis to generate the required ATP by increasing plasma membrane glucose transporter (Glut1) expression. A fraction of effector T cells differentiate into memory T cells which can survive for months to years. Memory T cells enhance mitochondrial biogenesis and switch to OXPHOS. However, exposure of the memory T cells to same antigen rapidly change them back to effector cells and adjusts their metabolism by shifting the metabolic pathway from OXPHOS to glycolysis [94].

1.9.3 Metabolic pathways in B lymphocytes

Metabolic pathways play a distinct role in B lymphocytes activation and differentiation. In contrast to T lymphocytes, B lymphocytes upregulate glycolysis and OXPHOS equally and

more broadly upon activation [95]. Metabolic reprogramming is unique for each stage of B cell differentiation. Naïve B cells are considered as quiescence cells which are slightly proliferate without activation; therefore, the metabolic requirements are very limited in these cells [96].

Activation of naïve B cells through the BCR leads to a marked increase in their metabolic pathways to generate ATP and meet energetic demands that are related to proliferation and maturation status. BCR-activated B cells require co-stimulatory signals such as co-stimulation through CD40 interaction in a T cell-dependent response or TLR engagement in a T cell-independent response [97, 98]. These secondary signals are needed to fully activate B cells. Previous studies showed that the absence of the secondary signals of B cells activation induces cell death by the accumulation of mitochondrial calcium and increased superoxide production [99]. After BCR stimulation and CD40 or TLR co-stimulation, B cells expand in balance both oxygen consumption and glucose uptake through increased Glut1 expression to enhance glycolysis and mitochondrial biogenesis. In addition, activated B cells increase glutaminolysis reactions and lipid synthesis. These metabolic changes are mainly dependent on PKCβ, mTOR activation, and mitochondrial function [95, 100–102].

Following TD antigen stimulation and help from $CD4^+$ T cell, germinal centers are formed, and B cells undergo marked changes in their proliferative and metabolic states. In GCs, B cells mature and differentiate into plasma cells and memory cells through somatic hypermutations (SHM) and class-switching recombination (CSR) [103]. The immunometabolism for GC B cells is critical and required for B-cell differentiation. Previous studies showed that GC B cells increase both mitochondrial mass and glucose uptake. BCR and CD40 signals in GC B cells are required for c-MYC induction. c-MYC is a transcription factor that regulates cell cycle and cellular metabolism, activates mTORC1, and drives the expression of enzymes and transporters that are required for glycolysis and glutaminolysis

[104, 105]. Another important enzyme for B cells in GC is glycogen synthase kinase 3 (GSK3) which acts as a metabolic sensor. GSK3 can protect B cells proliferation during c-MYC restriction and nutrient deprivation. Activated B cells in GC mediate GSK3 pathways to maintain GC B cells growth in a hypoxic microenvironment when glucose is restricted [106].

Plasma cells provide long immunity through antibodies production and secretion. Plasma cells produce thousands of antibodies per second. They undergo significant cellular alteration during proliferation and differentiation which enhance both glycolytic and oxidative metabolism. Glucose metabolism and OXPHOS are required for synthesis and secretion of antibodies in long-lived plasma cells (LLPCs) and short-lived plasma cells (SLPCs). BLIMP1, a transcription factor, plays an important role in plasma cells metabolic programming by increasing amino acid transporter expression for amino acids such as leucine and arginine which are used to promote protein synthesis. In addition, these amino acids activate the rapamycin complex 1 (mTORC1) which plays essential role in plasma secreting cells formation and antibody production. Additionally, plasma cells use amino acids for survival, antibody secretion, and energy production. For example, glutamine is utilized in OXPHOS to support ATP production and antibodies formation [107, 108]. Furthermore, plasma cells increase their spare respiratory capacity through increased pyruvate generation from the glycolysis pathway. Inhibition of pyruvate import into mitochondria *in vivo* in mice resulted in a decrease the spare respiratory capacity which lead to amelioration of vaccine-specific plasma cells production [109].

1.10 Mesenchymal stem cells

Bone marrow derived mesenchymal stem cells (MSCs) were first recognized by Friedenstein and colleagues in the early 1970s. These cells were identified *in vitro* as adherent, fibroblast-like nonphagocytic cells [110]. MSCs are considered as self-renewing and they are able to differentiate into osteocytes, adipocytes, and chondrocytes [111]. Additionally, MSCs

are characterized by expressing specific surface markers such as DC44, CD90, CD144, and CD166. However, they stain negative for the endothelial or hematopoietic surface markers such as CD14, CD19, CD34 and CD45 [112]. MSCs can be isolated from different sources including bone marrow, adipose tissue, placenta, umbilical cord, and fetal amniotic fluid [113]. Due to the unique characteristics of MSCs, MSCs have been recognized as a potential candidate in clinical applications for cellular therapy. MSCs are multipotent cells which can differentiate to specialized cells. In addition to self-renewal properties, MSCs can be isolated and easily expanded *ex vivo* without loss in their function. Furthermore, MSCs are considered immunomodulatory cells which can modulate the immune response through crosstalk with immunoregulatory cells and secretion of soluble factors. MSCs are also have the ability to migrate to injury and tumor sites [114].

1.10.1 Immunomodulatory effect of Mesenchymal stem cells

MSCs have significant immunosuppressive and anti-inflammatory properties and are able to interact with lymphocytes and modulate both the innate and adaptive immunity. MSCs can regulate the innate immune system by inhibiting the activation and differentiation of dendritic cells [115], suppressing the proliferation and cytokine secretion of natural killer cells [116], and inducing immunosuppressive M2 macrophage [117]. Moreover, MSCs can decrease T cells proliferation and potentiate the induction of regulatory T cells (Treg) phenotype which leads to an immunotolerant state [118]. Further, MSCs can potentiate the induction of plasmacytoid dendritic cells that may play a role to support Tregs development through interleukin (IL)-10 secretion [119]. In addition, MSCs can arrest the B cell cycle leading to suppression of B cell function and decreased antibodies secretion [120]. Although the MSCs can directly interact with immune cells through cell-to-cell contact, the main immunosuppressive mechanism of MSCs in through the production of several immunosuppressive soluble molecules such as indoleamine 2,3-dioxygenase (IDO), cytotoxic

T lymphocyte antigen 4 (CTLA-4), interleukin-10 (IL-10), nitric oxide, prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), and transforming growth factor-β (TGF-β) [121, 122] . Therefore, it has been suggested that MSCs are considered as a main regulator of the immune response through direct inhibition of activated immune cells and indirectly through Tregs recruitment.

The immunomodulatory effect of MSCs is largely dependent on local microenvironment structure and disease condition. For example, MSCs enhance the secretion of immunosuppressive molecules such as IDO, ICAM-1, and CCL-8 under acute inflammation when MSCs are polarized by M1 macrophages and type-1- helper T lymphocyte [119]. On the other hand, MSCs can inhibit the function of helper T1 lymphocytes in autoimmune diseases such as systemic lupus erythematosus (SLE) [123]. The inflammatory signals that emerge from inflammation and tumor microenvironments through cytokines, chemokines, and chemoattractant mediators trigger MSC migration. Therefore, MSCs migrate to inflammation and tumor sites through various cytokine and chemokines receptors such as stromal cell-derived factor 1 (SDF-1)/C-X-C chemokine receptor type 4 (CXCR4) [124], monocyte chemoattractant protein-1 (MCP-1)/C-C chemokine receptor type 2 [125], and vascular endothelial growth factor (VEGF)/VEGF receptor [126].

Several studies have reported that MSCs can suppress B cells proliferation, activation, and immunoglobulin secretion. Furthermore, MSCs can inhibit the B cells chemotactic properties through down-regulation of the expression of chemokine receptors in B cells including CXCR4, CXCR5 and CCR7 [120]. MSCs down-regulate the Blimp-1 mRNA expression in B cells which results in inhibition of B cells differentiation to secreting plasma cells [127]. Further, MSCs can arrest G0/G1 phases of B cells cycle through cytokines secretion which leads to inhibition of B cells proliferation [128]. In addition, MSCs can inhibit the

activation of complement system through expression of CD59 which lead to suppression of the activation of B cells complement system [129].

MSCs can induce and immunoregulate regulatory B cells (Bregs) via direct and indirect mechanisms including cell-cell contact, soluble factors secretion, and extracellular vesicles (EVs) [130]. Bregs exhibit immunosuppressive properties and modulate the immune response in patients with autoimmune diseases. Therefore, MSCs can synergize the immunosuppressive effect of Bregs and provide a promising therapy for immune-related diseases. MSCs are capable of inducing IL-10 producing regulatory B cells through the PD1-PDL1 pathway, which is considered the main pathway in cell-to-cell interaction between MSCs and B cells. Programmed death ligand-1 (PD-L1 or B7-H1) plays a vital role in suppressing adaptive immunity. Previous studies showed that MSCs express the PD-L1 on their surface to induce IL-10-producing Bregs [131]. In addition, MSCs can induce $CD23^{+}CD43^{+}$ Bregs via COX-2/PGE_2 pathway. It was reported that MSCs mediated $CD23^{+}CD43^{+}$ Bregs development through the COX-2/PGE_2 pathway resulted in a significant decrease in colon inflammation and reduced damage of gastrointestinal mucosal tissue in mice models [132]. Moreover, MSCs can upregulate of $CD5^{+}$ B cells phenotype through the IDO pathway by supporting the survival and proliferation of $CD5^{+}$ B cells [133]. $CD5^{+}$ B cells enhance immunosuppression through inhibition of the proliferation of T cells and promote Tregs formation (Figure 1.4) [134].

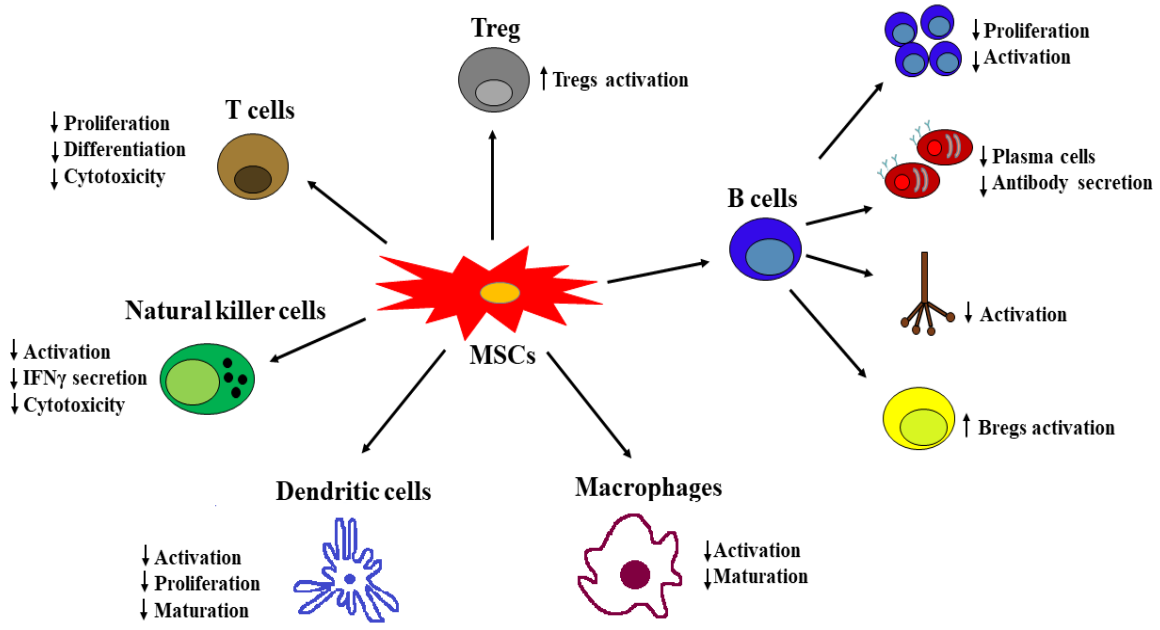

Figure 1.4 Immunosuppressive effect of MSCs. MSCs suppress adaptive and innate immune cells. MSCs can inhibit the activation and proliferation of B cells, decrease plasma cells differentiation and antibody secretion, inhibit complement system activation, and enhance Bregs activation. Furthermore, MSCs suppress T cells proliferation, differentiation and cytotoxicity, and increase Tregs formation. MSCs also inhibit macrophages activation and maturation, decrease dendritic cells activation, proliferation, and maturation, and suppress natural killer cells activation, cytotoxicity, and IFNγ production.

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CHAPTER II. Overall Rationale, Hypothesis and Objectives

2.1 Rationale: CL is a major membrane phospholipid of mitochondria. The appropriate content and acyl composition of CL is required for respiratory chain supercomplexes stability and ATP production in mitochondria [1, 2]. BTHS is a rare X-linked genetic disorder that is caused by mutation in the TAFAZZIN gene. The protein product Taz is a CL remodeling enzyme [3, 4]. Therefore, reduced CL remodeling in BTHS results in mitochondrial dysfunction. The mitochondrial dysfunction in BTHS patients results in metabolic abnormalities which are the main cause for the serious complications associated with the disease [5]. Immunodeficiency is one of the major life-threatening manifestations in BTHS patients that requires urgent intervention to avoid emergence of serious infections that may cause death [6]. The molecular mechanisms that lie behind this poor immunity are not fully understood. Therefore, in this thesis we investigated a possible association between Taz protein deficiency and B lymphocyte immune function. We also aimed to explore the effect of Taz deficiency on the immunoregulatory activity of mesenchymal stem cells (MSCs) since these cells may regulate B lymphocytes and immune system function. No previous studies had examined the impact of Taz deficiency on the immunological properties of B lymphocytes or the immunosuppressive properties of MSCs. Epstein-Barr virus transformed B lymphoblasts from BTHS patients and TazKD mice were used as experimental models for BTHS in our studies. We investigated the expression of surface markers in control and Epstein-Barr virus transformed BTHS lymphoblasts upon stimulation. Additionally, we investigated the impact of Taz deficiency in modulating naïve B cells metabolic and proliferative activities and immunological function upon stimulation. Since BTHS is considered a multi-system disorder, we additionally examined how Taz deficiency alters the immunosuppressive function of MSCs and how this impact B lymphocytes activity and phenotype.

2.2 Hypotheses:

1- We hypothesized that Taz deficiency in B lymphocytes results in a decreased mitochondrial function, and this impairs their ability to respond to activation.

2- We hypothesized that Taz deficiency increases the immunosuppressive effect of MSCs and this inhibits B lymphocyte proliferation and function.

2.3 Objectives:

1- To determine the effect of Taz deficiency on Epstein-Barr transformed BTHS lymphoblasts immunological function upon activation.

2- To investigate the role of Taz deficiency on murine TazKD B lymphocyte activation.

3- To characterize if Taz deficiency alters the immunosuppressive function of MSCs.

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CHAPTER III. Impaired surface marker expression in stimulated Epstein-Barr virus transformed lymphoblasts from Barth Syndrome patients

Rationale: The Epstein-Barr virus transformed BTHS lymphoblast *in vitro* model has been widely used to study the effect of Taz deficiency on CL composition and mitochondrial function. No previous studies had ever used this model to study the effect of Taz deficiency on B lymphoblasts immunological function. The underlying cause of immunodeficiency in BTHS patients is unknown. In the following section, we used Epstein-Barr virus transformed BTHS lymphoblasts to study the impact of Taz deficiency on the immunological activity of these cells upon stimulation. Our laboratory and others have reported that Epstein-Barr transformed BTHS lymphoblasts exhibit low CL levels, fragmented and poor cristae formation, and mitochondrial dysfunction. It is known that activation of B cells enhances their metabolic activities including OXPHOS and glycolysis to meet the cellular energy that is required for proliferation and differentiation. Therefore, in the following chapter, we determined how Taz deficiency may affect Epstein-Barr virus transformed BTHS lymphoblasts function after activation.

Hypothesis: Taz deficiency in Epstein-Barr transformed BTHS lymphoblasts alters their immunological function after stimulation.

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Impaired surface marker expression in stimulated Epstein-Barr virus transformed lymphoblasts from Barth Syndrome patients

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3.1 Abstract

Primary B lymphocytes rapidly respond to lipopolysaccharide (LPS) and cytosine linked to a guanine by a phosphate bond deoxyribonucleic acid (CpG DNA) stimulation to promote adaptive immune function through increased surface marker expression. Here we examined expression of surface markers in LPS and CpG DNA stimulated Epstein-Barr virus transformed B lymphoblasts from control and BTHS patients with different mutations. The percentage of cluster of differentiation (CD) positive cells including CD38+, CD138+, CD80+ surface expression and programmed cell death protein 1 (PD1+) surface expression was similar between control and BTHS lymphoblasts incubated plus or minus LPS. The percentage of CD24+, CD38+ and CD138+ cells was similar between control and BTHS lymphoblasts incubated plus or minus CpG DNA. CD27+ surface marker expression was reduced in both BTHS lymphoblasts and controls incubated with CpG DNA and PD1+ surface marker expression was higher in BTHS cells compared to controls but was unaltered by CpG DNA treatment. Thus, Epstein-Barr virus transformed control and BTHS lymphoblasts fail to increase selected surface markers upon stimulation with LPS and exhibit variable surface marker expression upon stimulation with CpG DNA. Since B lymphocyte surface marker expression upon activation is involved in B cell proliferation and differentiation, cell-cell interaction and the adaptive immune response, we suggest that caution should be exercised when interpreting immunological data obtained from Epstein-Barr virus transformed BTHS cells. Based upon our observations in control cells, our conclusions may be more broadly applicable to other diseases which utilize transformed B lymphocytes for the study of immune biology.

Key words: Barth Syndrome; B lymphoblasts; Tafazzin; lipopolysaccharide; cytosine linked to a guanine by a phosphate bond deoxyribonucleic acid; surface marker expression

3.2 Introduction

Barth Syndrome is a rare X-linked genetic disease caused by a mutation in the TAFAZZIN gene which codes for the cardiolipin (CL) transacylase protein tafazzin [1-3]. Tafazzin remodels nascent *de novo* synthesized CL into a form of CL found in the tissue specific mitochondrial membrane [4]. As the signature lipid of mitochondria, CL is required to support many mitochondrial functions including energy production required for adaptive immunity of B lymphocytes [5, 6].

Several laboratories, including our own, have utilized Epstein-Barr virus transformed B lymphoblasts from patients for the study of BTHS pathology [7-11]. The rationale for the use of these cells is that they are easy to maintain in culture and the transformed nature of these cells makes them readily amenable to experimental manipulation. In addition, they are representative of the specific mutation of a given patient. However, studies have also indicated that Epstein-Barr virus transformation of human B lymphocytes may hinder host immune function [12]. Epstein-Barr virus transformed B lymphoblasts express Toll-like receptors (TLRs) including TLR4 which can be stimulated with lipopolysaccharide (LPS) *in vitro* [13]. In addition, cytosine linked to a guanine by a phosphate bond deoxyribonucleic acid (CpG DNA) activates human B lymphocytes through TLR9 regardless of whether the DNA is in the form of genomic bacterial DNA or in the form of a synthetic oligodeoxynucleotide [14]. In this study, we examined whether Epstein-Barr virus transformed control and BTHS B lymphoblasts express surface markers indicative of activation by LPS and CpG DNA. We show that selected surface marker expression is refractory to stimulation with LPS and variable to stimulation with CpG DNA in Epstein-Barr virus transformed human B lymphoblasts from control and BTHS patients. Since B lymphocyte surface marker expression upon activation is required for B cell development, cell-cell interaction with other immune cells and the innate and humoral immune response, our results suggest that caution should be exercised when interpreting

immunological data obtained from Epstein-Barr virus transformed B lymphoblasts from BTHS patients.

3.3 Materials and Methods

Epstein-Barr virus transformed human control lymphoblasts and Epstein-Barr virus transformed BTHS lymphoblasts were obtained from the Coriell Institute for Medical Research (Camden, NJ, USA). The cells that were used in the study are outlined in Table 1. RPMI 1640 media, Fetal Bovine Serum (FBS), 1% Antibiotic-Antimycotic (A/A), and Propidium Iodide (PI) were obtained from Life Technologies Inc. (Burlington, ON, Canada). Anti-CD19-APC, anti-CD24-BV421, anti-CD27-perCP-CY5.5, anti-CD38-APC-H7, anti-CD138-PE, anti-CD80 PE-A, and anti-PD1-APC antibodies for flow cytometry analysis were purchased from BD Biosciences (Seattle, WA, USA). Unless otherwise indicated, all other reagents used were of analytical grade and were obtained from either Thermo Fisher Scientific (Winnipeg, MB) or Sigma-Aldrich (Oakville, ON).

3.1 Table 1

Cell lines used in this study:

Cell line (Identifier)	Phenotype	TAFAZZIN mutation	Age at harvest
07535	Healthy control	None	15 years
07491	Healthy control	None	17 years
16408	Healthy control	None	12 years
22192	BTHS	Exon 3, Substitution of cystine for arginine	10 years
22193	BTHS	Exon 6, premature stop codon	10 years
22194	BTHS	Complete deletion of TAFAZZIN gene	9 years

Cell culture and stimulation

All work was performed with approval from the University of Manitoba Environmental Health and Safety Office (Biological Safety Project Approval Certificate #BB0044-2). Cells were grown in RPMI-1640 medium supplemented with 15% FBS and 1% A/A at 5% CO_2 at 37°C in a Thermo Scientific CO_2 incubator HEPA Class 200. The medium was replaced every 48 h and the cells were passaged every five days. Cells were pelleted by centrifugation at 1400 rpm for 10 mins at room temperature. Cells were then washed twice with phosphate buffered saline (PBS) prior to experimental stimulation. Control and BTHS Lymphoblasts were incubated plus or minus 10 µg/ml lipopolysaccharide (LPS) (E. Coli 128: B12) for 24 h or 5 µM CpG DNA (ODN 2006) (TLR9 selective) for 24 h. After stimulation, the cells were harvested by centrifugation as above and washed twice with PBS prior to further analysis.

Cell viability and surface marker expression analysis

Briefly, after 24 h of stimulation with LPS or CpG DNA, the lymphoblasts were centrifuged at 1400 rpm for 10 min. The pellets were washed with PBS and suspended in 100 µl of PBS. Lymphoblasts were stained with propidium iodide (5 µg/ml) for 5 mins in the dark at 4°C. This was followed by flow cytometry analysis. Surface marker expression was measured in untreated and stimulated lymphoblasts by staining the cell surface with anti-CD19-APC, anti-CD24-BV421, anti-CD27-perCP-CY5.5, anti-CD38-APC-H7, anti-CD138-PE, anti-CD80 PE-A, and anti-PD1-APC as per the manufacturer's instructions. Cells were then analyzed by flow cytometry at the Flow Cytometry Core Facility in the Rady Faculty of Health Sciences, University of Manitoba, using a BD FACS Canto II instrument. FlowJo software was used for data analysis.

Statistical analysis

All data are expressed as mean ± SD. Comparison between two groups was performed by using Two tailed unpaired Student's t test, and comparison between multiple groups was achieved

using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test. A p value of < 0.05 was considered statistically significant.

3.4 Results

Cell viability is maintained in Epstein-Barr virus transformed human control and BTHS B lymphoblasts after stimulation

Initially we examined viability of LPS and CpG DNA treated control and BTHS B lymphoblasts. Epstein-Barr virus transformed B lymphoblasts from 3 control patients and 3 BTHS patients with different mutations were incubated for 24 h with 10 μg/ml LPS or 5 μM CpG DNA then stained with propidium iodide and flow cytometry performed to assess cell viability. We observed that cell viability was maintained in both control and BTHS lymphoblasts incubated with 10 μg/ml LPS (Fig. 1a) or 5 μM CpG DNA (Fig. 1b) for 24 h. Thus, cell viability is maintained in Epstein-Barr virus transformed human control and BTHS B lymphoblasts after stimulation with LPS or CpG DNA.

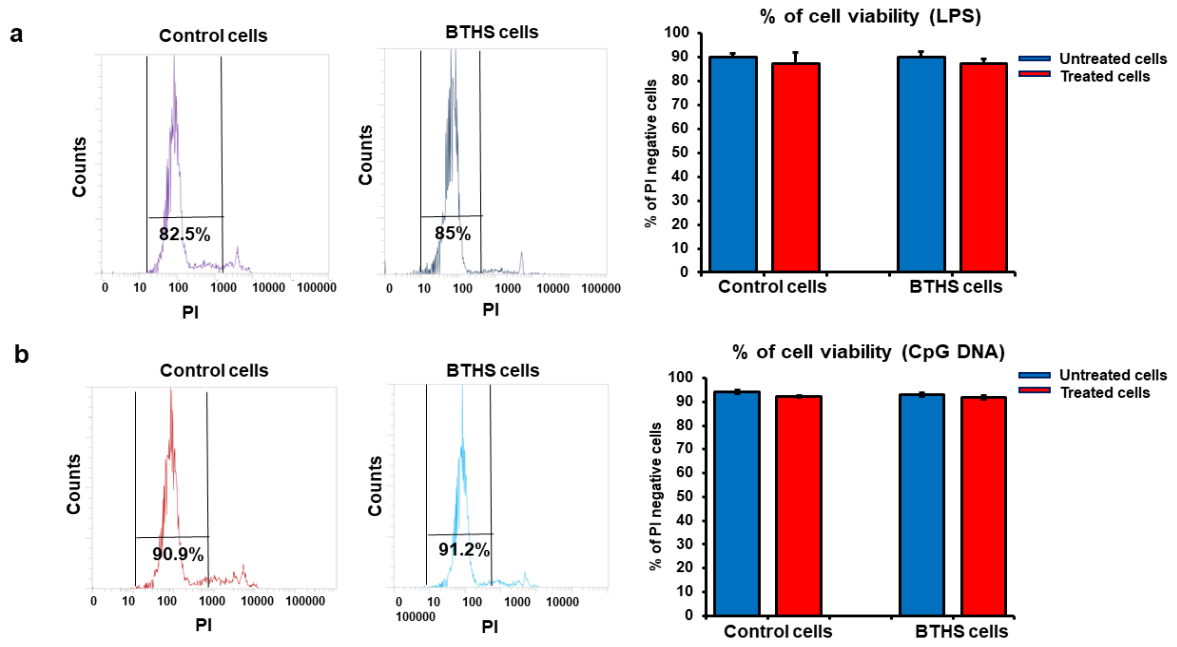


Figure 1. Cell viability is maintained in both Epstein-Barr virus transformed human control and BTHS lymphoblasts after stimulation. Cell viability was measured in control and BTHS lymphoblasts after stimulation with 10 µg/ml LPS or 5 µM CpG DNA using flow cytometry as described in Materials and Methods. **a.** Representative flow cytometry images of the percentage of cell viability in untreated and LPS treated cells. **b.** Representative flow cytometry images of the percentage of cell viability in untreated and CpG DNA treated cells. N=3.

Epstein-Barr virus transformed human control and BTHS B lymphoblasts surface marker expression is refractory to stimulation with LPS

Control and BTHS B lymphoblasts were incubated minus or plus 10 μg/ml LPS for 24 h and expression of the surface markers CD38+, CD138+, CD80+ and PD1+ determined. The percentage of CD38+ and CD138+ surface expression was similar between control and BTHS B lymphoblasts incubated minus or plus LPS for 24 h (Fig. 2 a, b & c). In addition, the percentage of CD80+ and PD1+ surface expression was similar between control and BTHS B lymphoblasts incubated minus or plus LPS for 24 h (Fig. 3 a, b & c). Thus, selected surface marker expression in Epstein-Barr virus transformed human control and BTHS B lymphoblasts was refractory to stimulation with LPS.

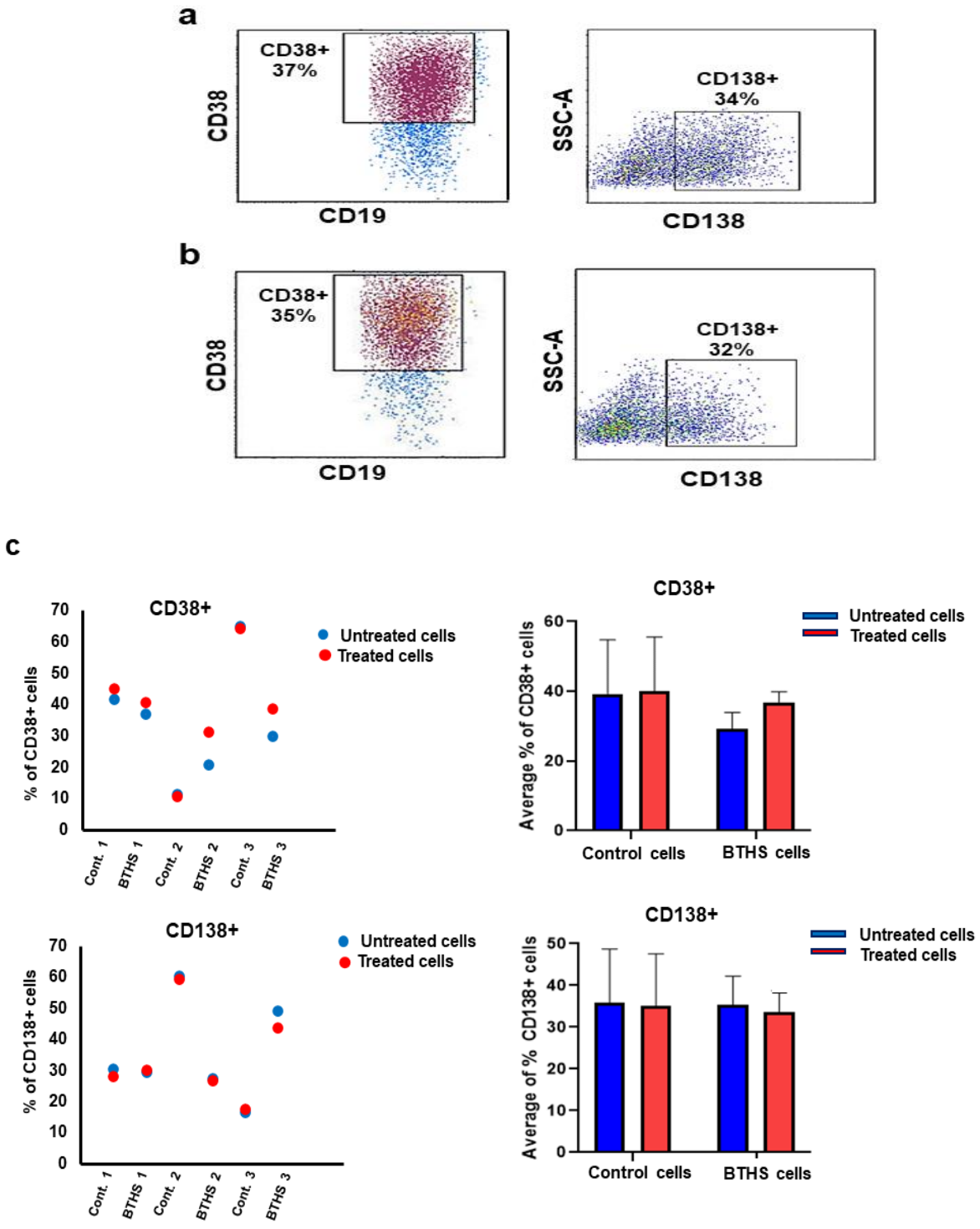


Figure 2. CD38+ and CD138+ surface markers expression in Epstein-Barr virus transformed human age-matched control and BTHS lymphoblasts after LPS stimulation. The expression of CD38+ and CD138+ surface markers were determined in control and BTHS lymphoblasts after stimulation without or with 10 μg/ml LPS using flow cytometry as

described in Materials and Methods. **a.** Representative flow cytometry images of CD38+ and CD138+ surface markers in control cells after LPS stimulation. **b.** Representative flow cytometry images of CD38+ and CD138+ surface markers in BTHS cells after LPS stimulation. **c.** Histograms showing percentage of CD38+ and CD138+ surface markers expression in LPS treated control and BTHS cells compared to untreated control and BTHS cells. N=3.

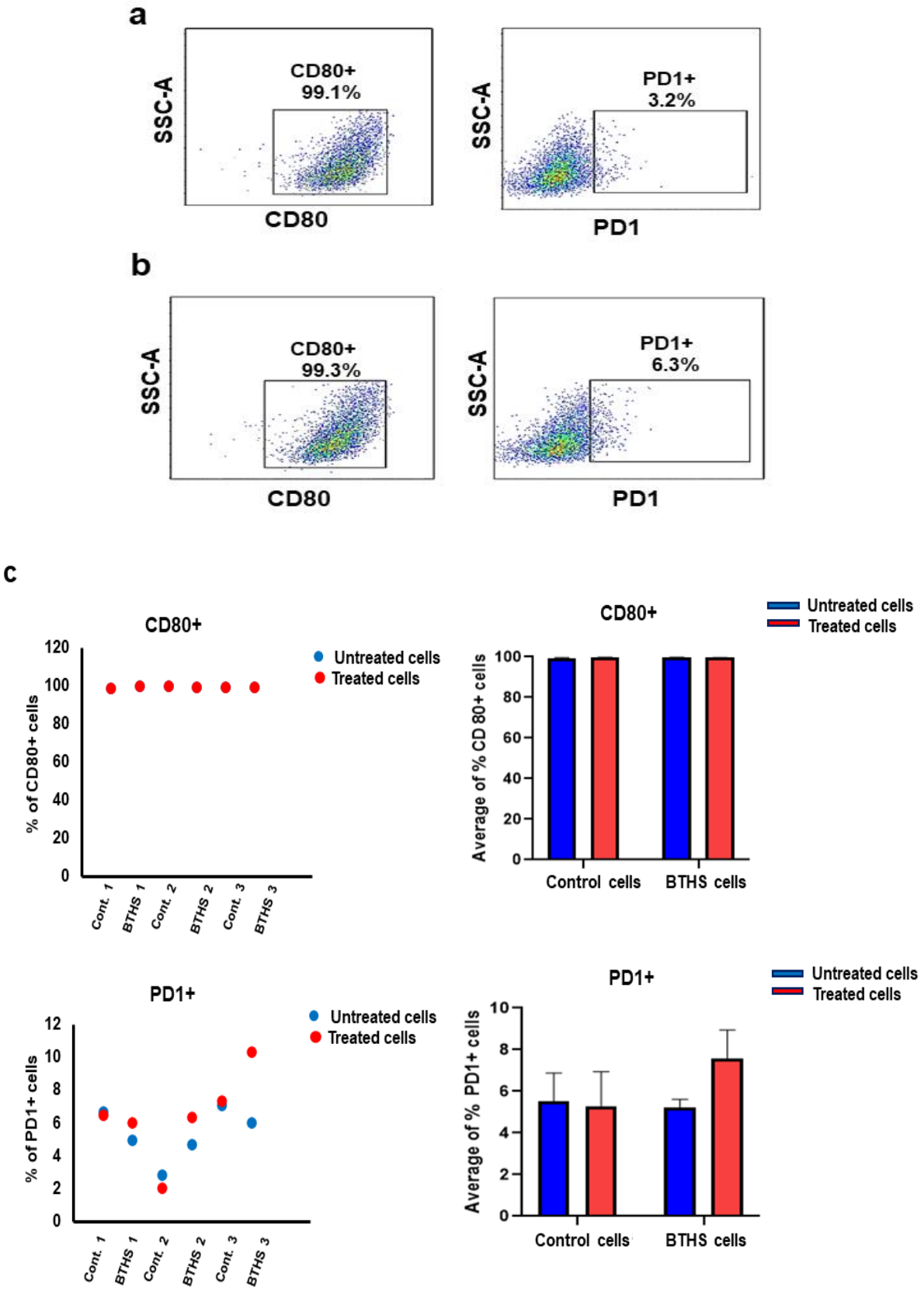
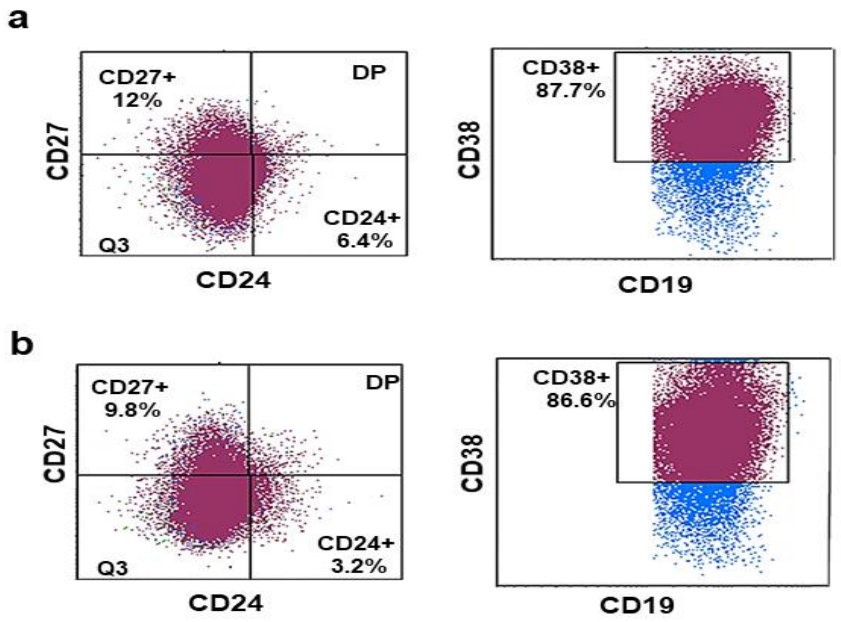


Figure 3. CD80+ and PD1+ surface markers expression in Epstein-Barr virus transformed human age-matched control and BTHS lymphoblasts after LPS stimulation.

The expression of CD80+ and PD1+ surface markers were determined in control and BTHS lymphoblasts after stimulation without or with 10 μg/ml LPS using flow cytometry as described in Materials and Methods. **a.** Representative flow cytometry images of CD80+ and PD1+ surface markers in control cells after LPS stimulation. **b.** Representative flow cytometry images of CD80+ and PD1+ surface markers in BTHS cells after LPS stimulation. **c.** Histograms showing percentage of CD80+ and PD1+ surface markers expression in LPS treated control and BTHS cells compared to untreated control and BTHS cells. N=3.

Epstein-Barr virus transformed human control and BTHS B lymphoblasts show variable surface marker expression upon CpG DNA stimulation

Control and BTHS B lymphoblasts were incubated minus or plus 5 μM CpG DNA for 24 h and expression of the surface markers CD24+, CD38+, CD138+, CD27+ and PD1+ determined. The percentage of CD24+ and CD38+ was similar between control and BTHS B lymphoblasts incubated minus or plus 5 μM CpG DNA for 24 h (Fig. 4a, b & c). In contrast, CD27+ surface marker expression was reduced in both control and BTHS B lymphoblasts incubated with 5 μM CpG DNA for 24 h (Fig. 4a, b & c). The percentage of CD138+ expression was similar between control and BTHS B lymphoblasts incubated minus or plus 5 μM CpG DNA for 24 h (Fig. 5a, b & c). In contrast, the percentage of PD1+ expression was higher in BTHS cells compared to controls but was unaltered by incubation with 5 μM CpG DNA for 24 h (Fig. 5a, b & c). Thus, Epstein-Barr virus transformed human control and BTHS B lymphoblasts showed variable selected surface marker expression upon CpG DNA stimulation.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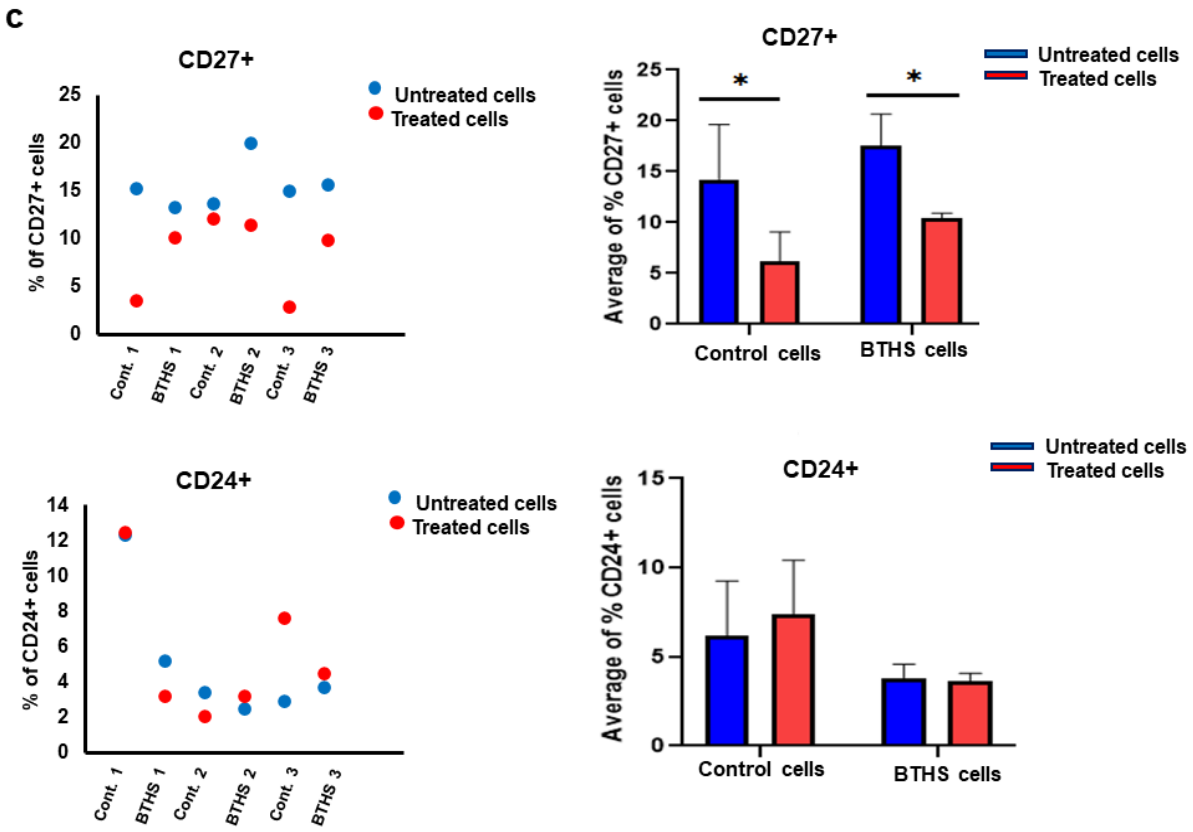
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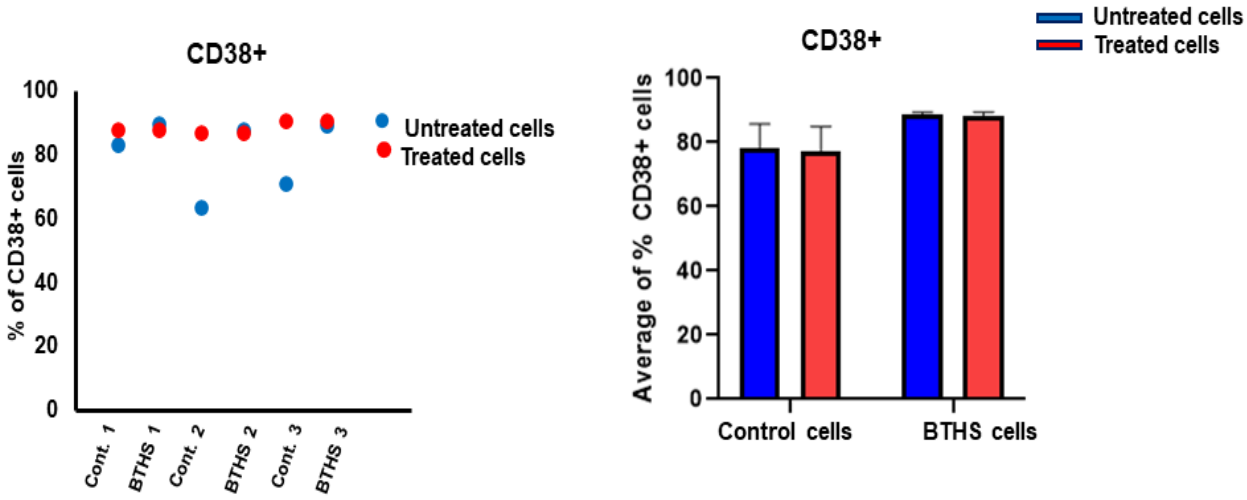

Figure 4. Epstein-Barr virus transformed human age-matched control and BTHS B lymphoblasts show variable expression of CD27+, CD24+, and CD38+ surface markers upon CpG DNA stimulation. The expression of CD27+, CD24+, and CD38+ surface markers was determined in control and BTHS cells after stimulation without or with 5 μM CpG DNA using flow cytometry as described in Materials and Methods. **a.** Representative flow cytometry images of CD27+, CD24+, and CD38+ surface markers in control cells after CpG DNA stimulation. **b.** Representative flow cytometry images of CD27+, CD24+, and CD38+ surface markers in BTHS cells after CpG DNA stimulation. **c.** Histograms showing percentage of CD27+, CD24+, and CD38+ surface marker expression in CpG DNA treated control and BTHS cells compared to untreated control and BTHS cells. N=3, *$p<0.05$.

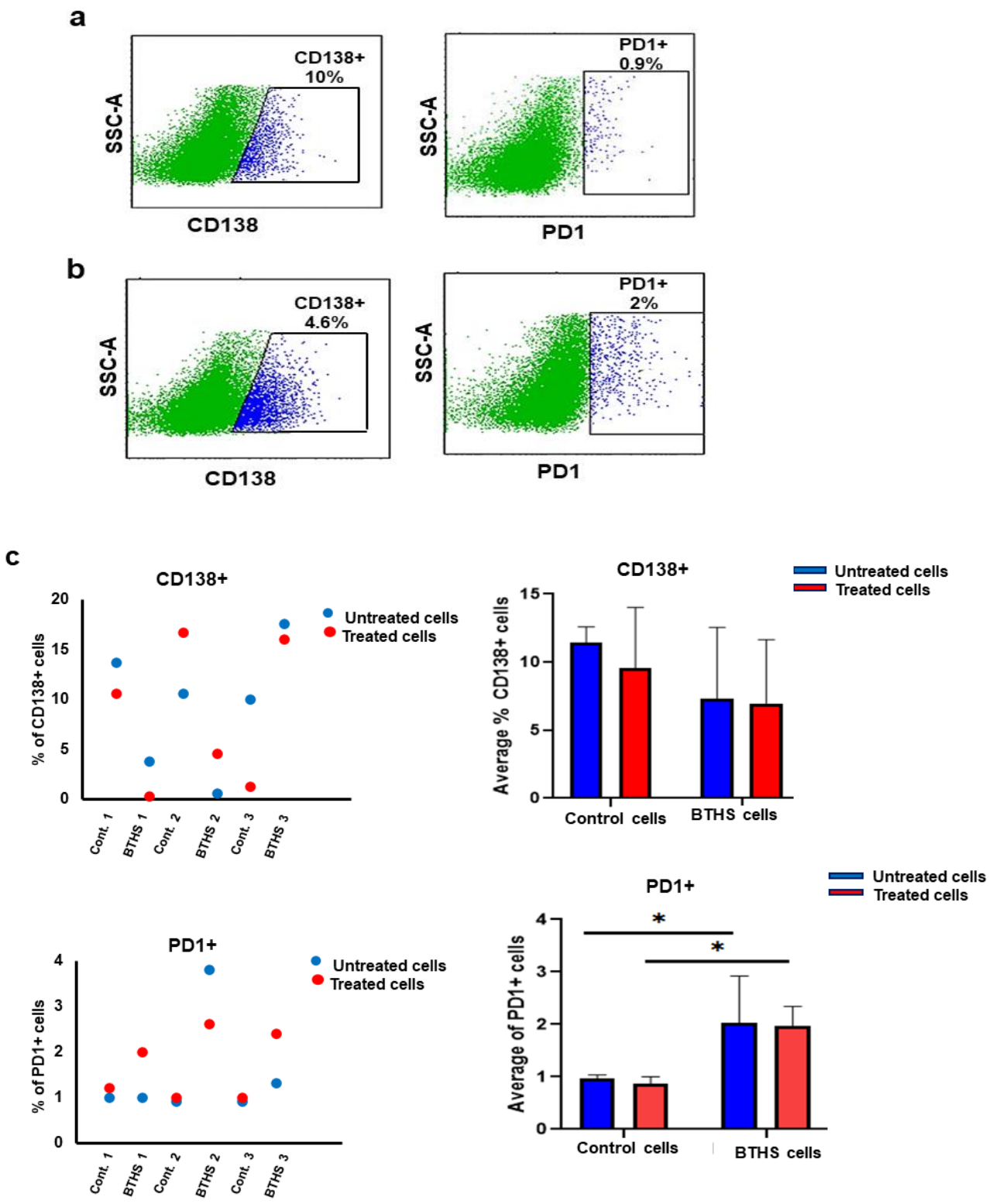

Figure 5. CD138+ and PD1+ surface markers expression in Epstein-Barr virus transformed human age-matched control and BTHS lymphoblasts after CpG DNA stimulation. The expression of CD138+ and PD1+ surface markers were determined in control

and BTHS cells after stimulation without or with 5 μM CpG DNA using flow cytometry as described in Materials and Methods. **a.** Representative flow cytometry images of CD138+ and PD1+ surface markers in control cells after CpG DNA stimulation. **b.** Representative flow cytometry images of CD138+ and PD1+ surface markers in BTHS cells after CpG DNA stimulation. **c.** Histograms showing percentage of CD138+ and PD1+ surface marker expression in CpG DNA treated control and BTHS cells compared to untreated control and BTHS cells. N=3, *$p<0.05$.

3.5 Discussion

In this study we examined whether Epstein-Barr virus transformed control and BTHS B lymphoblasts expressed surface markers indicative of classical B lymphocyte activation upon LPS or CpG DNA stimulation. Surprisingly, our results clearly demonstrate that Epstein-Barr virus transformed human control and BTHS B lymphoblasts are refractory to selected surface marker expression upon stimulation with LPS and exhibit variable selected surface marker expression upon stimulation in response to CpG DNA.

CD24 is expressed at the surface of activated B lymphocytes and is required for B cell development and plays a key role in cell-cell adhesion [15]. In addition, CD24 expression on B cells is required for T cell co-stimulation in CD4 T-cell mediated clonal expansion [16]. CD38 is a key surface marker for B cell activation and plays an important role in B cell proliferation and regulation [17]. Previous studies showed that deficiency of CD38 expression resulted in inhibition of the immune response and increased susceptibility to infections [18]. CD138 is a specific surface marker of plasma cells which are required for long-term humoral immunity [19]. Upon stimulation, B cells differentiate into antibody secreting cells and upregulate CD138 on their surface which enhances antibody secreting cell maturation and accumulation [20]. CD80 is expressed in activated B cells and provides a co-stimulatory signal necessary for T cell activation [21]. A previous global gene expression profile analysis demonstrated significant differences in the gene expression patterns between Epstein-Barr virus transformed and normal host lymphocytes and that transformation may hinder normal immune function [12]. We recently demonstrated that isolated naïve primary murine B lymphocytes stimulated with LPS readily increased surface marker expression and that tafazzin deficiency in these cells markedly impaired surface marker expression upon stimulation with LPS [22]. The lack of elevated surface marker expression in human control and BTHS lymphoblasts in response to LPS, coupled with the fact that these surface markers were readily

detectable in unstimulated cells, indicate that these cells exhibit impaired responsiveness to LPS-mediated activation.

CD27 is a B lymphocyte memory marker and a co-stimulatory immune molecule which belongs to the tumor necrosis factor receptor superfamily [23]. CD27 surface marker expression plays an important role in immunoglobulin synthesis and in regulating B cell activation through binding with CD70 on activated B and T lymphocytes and modulates induction of apoptosis [24, 25]. PD1 surface marker expression down regulates the immune system to limit inflammation by modulating T-cell activity [26]. For example, PD1 expressed on the surface of activated B cells inhibited CD4+ and CD8+ T cell proliferation [27]. In addition, inhibition of the PD1 pathway resulted in increased B cell activation, proliferation and secretion of inflammatory cytokines [28]. Intriguingly, CD27 surface expression was reduced in both control and BTHS B lymphoblasts stimulated with CpG DNA. In contrast, PD1 surface expression was higher in BTHS cells compared to controls but was unaltered by stimulation with CpG DNA. These data suggest that Epstein-Barr virus transformed human control and BTHS B lymphoblasts exhibit variability in surface marker expression upon CpG DNA stimulation.

The significance of our observations is currently unknown. However, Epstein-Barr virus infection of human B lymphocytes was recently shown to promote major modifications of alternative splice variant expression which may impact on cell fate determination [29]. In addition, the differences in gene expression patterns observed between Epstein-Barr virus transformed and normal lymphocytes [12, 30], coupled with our observation of impaired B cell surface marker expression, could potentially influence B lymphocyte function when infection is mimicked through LPS- or CpG DNA-mediated activation. Thus, the Epstein-Barr virus transformed BTHS lymphoblast phenotype may exhibit a lack of responsiveness to pro-inflammatory stimuli and potentially attenuate activation of other immune cells. For example,

activated B lymphocytes secret CXCL1 and GRO-chemokines which recruit neutrophils to sites of infection and Epstein-Barr virus infection downregulates the expression of CXCL1 [12]. Hence, impaired B cell activation of Epstein-Barr virus transformed BTHS lymphoblasts could potentially attenuate neutrophil recruitment. Another conclusion from our study, derived from the behavior of the controls, is that it can be assumed that the effects are not necessarily limited to BTHS but are more broadly applicable to other diseases which utilize Epstein-Barr virus transformed B lymphocytes for the study of immune pathology. In summary, based upon our observations we suggest that caution should be exercised when interpreting immunological data obtained from Epstein-Barr virus transformed lymphoblasts.

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Informed Consent

Not applicable

Conflict of interests

The authors of this study have no financial and non-financial conflict of interest.

Author contributions

HMZ performed the experiments and analyzed the data. HMZ and GMH were responsible for conceptual design and wrote the manuscript. The authors have read and approved the final version of the manuscript.

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CHAPTER IV. Tafazzin deficiency impairs mitochondrial metabolism and function of lipopolysaccharide activated B lymphocytes in mice

Rationale: In chapter II, we found that both control and Epstein-Barr virus transformed BTHS lymphoblasts are refractory to LPS-mediated stimulation of surface marker expression. Additionally, stimulation of control and Epstein-Barr virus transformed BTHS lymphoblasts with CpG DNA resulted in variable surface marker expression. Expression of surface markers is considered an early sign of B cell activation. We found that immortalized Epstein-Barr virus transformed BTHS lymphoblasts exhibited different phenotypic features than that of naïve B cells. Therefore, in this chapter, we used the doxycycline-inducible TazKD mouse model to investigate the impact of Taz deficiency on the ability of B cells to be stimulated. We isolated naïve B lymphocytes from the spleen of WT and TazKD mice and then stimulated them with LPS and examined their immunological properties. Since BTHS is considered a multisystem metabolic disorder, understanding the impact of Taz deficiency in B lymphocyte function and development may provide a better understanding of why some BTHS patients exhibit poor immunity.

Hypothesis: Taz deficiency in B lymphocytes results in a reduction in metabolic activities and cellular function upon LPS stimulation.

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Tafazzin deficiency impairs mitochondrial metabolism and function of lipopolysaccharide activated B lymphocytes in mice

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Running Title: Tafazzin regulates activated B lymphocyte function

Key words: Tafazzin, B lymphocyte activation, mitochondria, lipopolysaccharide, cardiolipin, proteasome, immunoproteasome, cytokines, immunoglobulin synthesis and secretion, cell proliferation and survival

4.1 Abstract

B lymphocytes are responsible for humoral immunity and play a key role in the immune response. Optimal mitochondrial function is required to support B cell activity during activation. We examined how deficiency of tafazzin, a cardiolipin remodeling enzyme required for mitochondrial function, alters the metabolic activity of B cells and their response to activation by lipopolysaccharide in mice. B cells were isolated from 3-month-old wild type or tafazzin knockdown mice and incubated for up to 72 h with lipopolysaccharide and cell proliferation, expression of cell surface markers, secretion of antibodies and chemokines, proteasome and immunoproteasome activities, and metabolic function determined. In addition, proteomic analysis was performed to identify altered levels of proteins involved in survival, immunogenic, proteasomal and mitochondrial processes. Compared to wild type lipopolysaccharide activated B cells, lipopolysaccharide activated tafazzin knockdown B cells exhibited significantly reduced proliferation, lowered expression of cluster of differentiation 86 and cluster of differentiation 69 surface markers, reduced secretion of immunoglobulin M antibody, reduced secretion of keratinocytes-derived chemokine and macrophage-inflammatory protein-2, reduced proteasome and immunoproteasome activities, and reduced mitochondrial respiration and glycolysis. Proteomic analysis revealed significant alterations in key protein targets that regulate cell survival, immunogenicity, proteasomal processing and mitochondrial function consistent with the findings of the above functional studies. The results indicate that the cardiolipin transacylase enzyme tafazzin plays a key role in regulating mouse B cell function and metabolic activity during activation through modulation of mitochondrial function.

4.2 Introduction

Proper functioning of B lymphocytes is essential to establish a normal immune response [1]. Naïve B cells proliferate and differentiate upon activation in order to provide humoral

responses and long-term immunity. Lipopolysaccharide (LPS) is a gram-negative bacterial product that can activate murine B cells through binding to the Toll-like receptor-4 [2]. Activation of B cells with LPS induces B cell proliferation, chemokines synthesis and secretion and production of antibodies [3, 4]. Normal metabolic activity in B lymphocytes is critical for B cell homoeostasis and immune responses. Importantly, B lymphocytes increase oxygen consumption and lactate production as a response to activation and this is required to support proper B cell function. For example, activation of murine B cells with LPS has been shown to increase both mitochondrial oxidative phosphorylation and glycolysis [3].

Tafazzin (Taz) is a transacylase enzyme involved in remodeling and maturation of the mitochondrial phospholipid cardiolipin (CL) [5]. CL is an essential phospholipid of the inner mitochondrial membrane where it comprises approximately 15-20% of inner mitochondrial membrane phospholipid mass [6]. CL is required for activation of many enzymes of the electron transport chain [7]. Mutations in the tafazzin gene (TAFAZZIN) are observed in the rare (1:300,000) X-linked genetic disease Barth Syndrome (BTHS) [8, 9]. BTHS patient cells exhibit reduced ability to produce ATP from oxidative phosphorylation which leads to the dysfunction of metabolically active tissues such as the heart and skeletal muscle [10]. Although cardiomyopathy is the major clinical problem in BTHS, many of these patients additionally suffer from severe infections caused by neutropenia [8, 9].The molecular mechanisms underlying this neutropenia remain unclear. Interestingly, neutrophils from BTHS patients exhibit normal motility, phagocytosis, mitochondrial shape and mass, with increased annexin-V binding in the absence of apoptosis [11]. It has also been observed that a few BTHS patients exhibit infections despite consistent prevention of neutropenia. Thus, it is possible that defects in other immune cell types may additionally contribute to the infections in BTHS. Although B cells play a key role in the development of the immune response to infection, no studies have examined how Taz-deficiency impacts on B lymphocyte proliferation and function during their

activation. B lymphocyte dysfunction may contribute to immune system dysfunction and potentially increase susceptibility to infection [12]. In addition, it is well established that B cells play a critical role in neutrophil recruitment, activation of other immune cells including T cells, and long-term immune protection [4, 13]. Previous studies have indicated that Epstein-Barr virus immortalized B lymphoblasts from BTHS patients exhibit increased mitochondrial size/mass, reduced cristae formation, variable cristae distribution, destabilization of supercomplexes, and elevated reactive oxygen species production [14–18]. It was hypothesized that a compensatory increase in mitochondrial content prevents a decrease in respiration and ATP synthesis in these cells [15]. However, the limitation of using these human Epstein-Barr virus transformed lymphoblasts for BTHS immune function studies is that the cellular responses to treatments differ markedly between normal lymphocytes and these cells due to the viral-induced cellular changes in the transformed cells [19].

In the current study, we determined how Taz-deficiency impacts on the function of LPS stimulated B cells. In addition, we identify novel protein targets involved in the Taz-mediated regulation of LPS stimulated B cell function.

4.3 Material and Methods

Experimental animals

All experimental procedures in this study were performed with approval of the University of Manitoba Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines and regulations. This study is reported in accordance with ARRIVE guidelines. All animals were housed in pathogen-free facility (12 h light/dark cycle) at the University of Manitoba and had free access to food and water. Tafazzin knockdown (TazKD) mice were generated by mating transgenic male mice containing a doxycycline (dox) inducible tafazzin specific short-hair-pin RNA (B6.Cg-Gt(ROSA)26Sortm1(H1/tetO-RNAi:Taz,CAG-

tetR)Bsf/ZkhuJwith female C57BL/6J mice from Jackson Laboratory (Boston, MA). Tafazzin knock down was maintained postnatally by administering dox (625 mg of dox/kg of chow) as part of the rodent chow (Catalog no. TD.01306) from Harlan (Indianapolis, USA) as described previously[20]. Male offspring were weaned at 3 weeks of age onto the above diet and wild type (WT) animals additionally received the dox containing diet. Male mice were used for the experiments between 10 and 14 weeks of age. Male mice positive for the tafazzin shRNA transgene were identified by PCR as described previously [20].

Cell culture

Splenic naïve B lymphocytes were isolated by magnetic bead negative selection using EasySep^{TM} Mouse B Cell Isolation Kit (Catalog no. 19854) from STEMCELL Technologies (Vancouver, BC). B cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% antibiotic/antimycotic, and 2-mercaptoethanol at 37°C with 5% CO_2. Subsequent to isolation B cells were stimulated or not with 10 µg/ml LPS (Catalog no. L2887) obtained from Sigma-Aldrich (Oakville, ON). Unless otherwise indicated, all other reagents used were of analytical grade and were obtained from either Thermo Fisher Scientific (Winnipeg, MB) or Sigma-Aldrich (Oakville, ON).

Surface marker analysis

The expression of B cell surface markers cluster of differentiation 86 (CD86) and cluster of differentiation 69 (CD69) were measured using flow cytometry. B cells (2×10^6) were cultured in 1 ml/well in 24-well plate in the presence 10µg/ml LPS at 37°C in 5% humidified CO_2 for 24 h. Subsequently, the B cells were stained with APC-conjugated anti-cluster of differentiation 19 (CD19) in combination with either PE-A labelled anti-CD69 or PE-CY7-A labelled anti-CD86. Anti-CD19, anti-CD69, anti-CD86 were obtained from BD Biosciences

(Mississauga, ON). Cells were analyzed by flow cytometry using a BD FACSCanto II instrument. FlowJo software was used for data analysis.

Analysis of proliferating and apoptotic cells

Proliferating and apoptotic cells were determined by cell cycle analysis using the Nicoletti assay [21]. B cells were treated with 10 µg/ml LPS for 48 h and cultured in 24-well plates. The cells were washed two times with PBS and suspended in 100 µl of ice-cold PBS. Cells were then fixed in 5 ml of 70% ice-cold ethanol. After fixation the cells were stained with DNA fluorescent dye that quantitatively stains the DNA (40 µg/ml propidium iodide, 0.5 mg/ml RNase A, 0.1% Triton X-100, 1% sodium citrate) for 1 h at 4°C in the dark. Then flow cytometry analysis was performed to detect red fluorescence (460 nm) for 10,000 cells using an Attune NxTModel AFC2 flow cytometer (Thermo Fisher Scientific). The number of proliferating cells was determined by counting the number of cells entering the S/G2 phase from the G0/G1 phase (non-proliferating phase). The percentage of apoptotic nuclei in sub-G1 section, healthy nuclei in G0/G1 section, and proliferating nuclei in S/G2 section were estimated by analysis of the DNA histogram and statistically compared between the samples [21].

Measurement of chemokines and antibodies

Purified B cells were incubated in RPMI 1640 media supplemented with FBS, 2-mercaptoethanol, and 1% antibiotic/antimycotic. B cells were stimulated with 10 µg/ml LPS at 37°C with 5% CO_2. After incubation for 24 h or 72 h, the supernatant fractions were collected to measure chemokines or antibodies concentrations, respectively. Immunoglobulin G (IgG), Immunoglobulin M (IgM), and the neutrophil attractant chemokines keratinocytes-derived chemokine(KC) and macrophage-inflammatory protein-2 (MIP-2) levels in cell culture supernatants were measured using isotype-specific enzyme-linked immunosorbent assay (ELISA) kits. IgG (total) mouse uncoated ELISA kit (Catalog no.88-50400-22) and IgM mouse

uncoated ELISA kit (Catalog no.88-50470-22) were from Thermo Fisher Scientific (Winnipeg, MB). Mouse CXCL2/MIP-2 DuoSet ELISA (Catalog no. DY452-05) and mouse CXCL1/KC DuoSet ELISA (Catalog no. DY453-05) were obtained from R&D Systems (Minneapolis, MN).

Proteasome and immunoproteasome activity assays

Immunoproteasome activity was determined by measuring the proteolytic activity of β1i/LMP2with S310 (Ac-PAL-AMC) as a fluorescent substrate using a commercial kit (Catalog no. 10008041) from Cayman Chemicals (Ann Arbor, MI). The fluorescent intensity was read at excitation = 345nm and emission=445nm. To measure proteasome activity, the 20S subunit activity was determined using fluorescent substrate-SUC-LLVY-AMC (Catalog no. 10008041) from Cayman Chemicals (Ann Arbor, MI). The fluorescent intensity was read at excitation=360 nm and emission=480nm.

Metabolic assays

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured in B cells using a Seahorse XF24 Extracellular Flux Analyzer (Agilent Technologies) with kits from Agilent (Santa Clara, CA). Briefly, isolated WT and TazKD B cells were stimulated with LPS for 48h. B cells were then seeded in XF24 24-well plates coated with Cell-Tak from BD Biosciences (Mississauga, ON) at a density of 4×10^5 cells per well. The Cell-Tak (1.12 μg/well) was used to generate an adherent monolayer of B cells on the XF24 24-well plate. For mitochondrial function stress test (OCR assay) the following final concentrations of drugs were used: 1 μM oligomycin, 1 μM FCCP, 1 μM antimycin A, and 1 μM rotenone as per the manufacturer's instructions. For glycolytic function test (ECAR assay) the following final concentrations of drugs were used: 10 μM glucose, 1 μM oligomycin, and 25 μM 2-DG as per the manufacturer's instructions. The data was normalized to the cell number in each well.

Western blot analysis

Cell proteins were extracted using RIPA lysis buffer (Catalog no. 89900) from Thermo Fisher (Winnipeg, MB). The total protein levels were measured by the Bradford method [22]. Briefly, 30µg of protein was mixed with an equal volume of 2×Laemmli buffer supplemented with 5% 2-mercaptoethanol (Catalog no. 1610737) then loaded in 10% polyacrylamide precast gel (TGX™ FastCast™ Acrylamide Starter Kit, 10%, Catalog no. 1610172) from BioRad Laboratories (Mississauga, ON). After 1 h electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane at 4°C. This was followed by blocking the membrane using 5% milk in a solution of TBS containing 0.5% Tween 20 at room temperature for 1 h. Then, the membrane was washed with 0.5% Tween 20 three times and incubated with primary antibody overnight at 4°C. The primary antibody was removed the next day and the membrane was incubated with secondary antibody at room temperature for 1 h. Enhanced chemiluminescence substrate (Clarity Western ECL Substrate, Catalog no.1705060) from Bio-Rad Laboratories (Mississauga, ON) was used to visualize the protein bands. The primary antibodies used for Western blotting were as follows: Tafazzin (Catalog no. sc-365810), PI 3-kinase p85α (Catalog no. sc-1637), AKt (Catalog no. sc-5298), pAKt (Catalog no. sc-293125), Mouse complexes antibody- Total OXPHOS Rodent WB Antibody Cocktail (Catalog no. ab110413, abcam), BTLA (Catalog no. Catalog # AF3007-SP, PSMA4 (Catalog no. ab191403), Aven (Catalog no. 2300), NDUFV1 (Catalog no. PA5-115633), MTCO1 (Catalog no. ab14705, abcam) and β-Actin (Catalog no. sc-47778) from Santa Cruz Biotechnolgies Inc. (Dallas, TX).Band intensity was quantified using ImageJ software.

Proteomic analysis

Cell lysis protocol: B cells were resuspended in lysis buffer (100mM HEPES, pH 8.5, 4% SDS, 1X protease inhibitors) and sonicated three times for 15 s per cycle with a 1-minute cooling

period (on ice) between each cycle. Then cell lysates were centrifuged at 15000g for 10 mins to remove insoluble cellular debris.

Protein Digestion and Tandem Mass Tag (TMT) labeling: The Pierce detergent compatible Bradford assay kit from Thermo Fisher (Winnipeg, MB) was used to determine the protein concentration. All protein samples were processed and handled using single-pot solid-phase-enhanced sample preparation protocol. Prior to the sample preparation protocol treatment, two types of carboxylate-modified SeraMag Speed beads from GE Life Sciences (Mississauga, ON) were combined in a ratio of 1:1 (v/v), rinsed, and reconstituted in water at a concentration of 20 µg solids/µL. Initially, 100 µg of lysate was reduced with 10mM (final concentration) DTT for 30 minutes at 60˚C followed by alkylation using 50mM (final concentration) IAA for 45 mins in dark at room temperature. After that, 10 µL of the prepared bead mix was added to the lysate and samples were adjusted to pH7.0 using HEPES buffer. Acetonitrile was added to a final concentration of 70% (v/v) to promote proteins binding to the beads. Samples were then incubated at room temperature on a tube rotator for 18 min. Subsequently, beads were immobilized on a magnetic rack for 1 min. The pellet was rinsed 2X with 200 µl of 70% ethanol and 1X with 200 µl of 100% acetonitrile while on the magnetic rack and the supernatant was discarded. Rinsed beads were resuspended in 40 µL of 50 mM HEPES buffer, pH8.0, supplemented with trypsin at an enzyme-to-protein ratio of 1:25 (w/w) and incubated for 16 h at 37˚C. After overnight digestion, peptides were eluted and immediately labeled with TMT tags. After overnight digestion, peptides were eluted and immediately labeled with TMT tags. TMT labeling was performed as specified by the manufacturer's instructions (Thermo Fisher, Winnipeg, MB), except that TMT tags were dissolved in DMSO. Equivalent labeled samples within each TMT set were mixed prior to 1D LC/MS/MS.

Mass spectrometry data acquisition: Analysis of TMT labeled peptide digests was carried out on an Orbitrap Q Exactive HF-X instrument from Thermo Fisher Scientific (Bremen, DE). The

sample was introduced using an Easy-nLC 1000 system from Thermo Fisher Scientific (Bremen, DE) at 2 μg per injection. Data acquisition on the Orbitrap Q Exactive HF-X instrument was configured for data-dependent method using the full MS/DD-MS/MS setup in a positive mode.

Statistical analysis

All experimental results were expressed as mean ± SD. The levels of significance were calculated using two tailed unpaired Student's t test unless otherwise specified one-way-analysis of variance followed by multiple comparisons using the Tukey's test. A p value of <0.05 was considered statistically significant.

4.4 Results

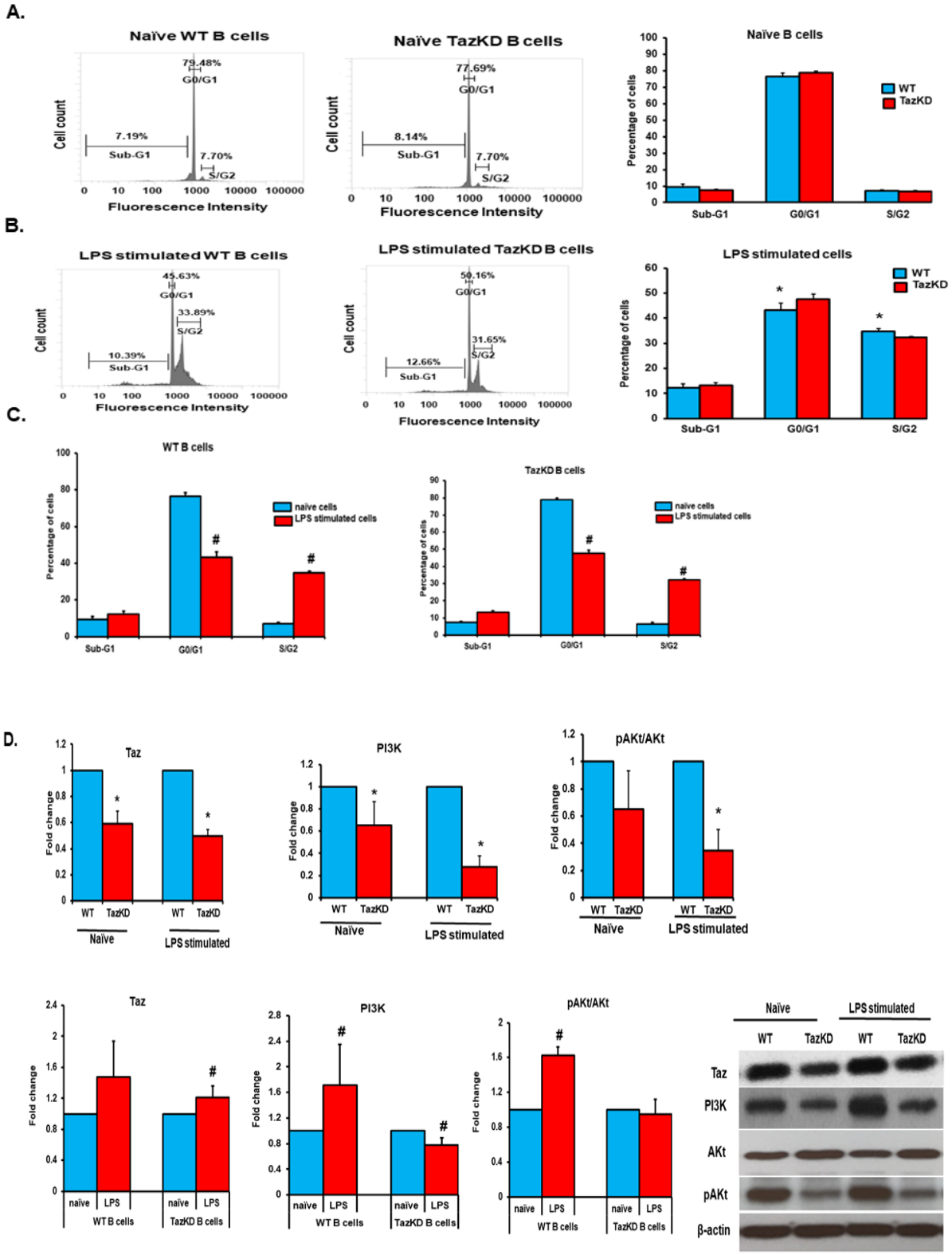
TAZ-deficiency decreases proliferation and reduces survival markers in LPS stimulated B cells

The percentage of cells in the Sub-G1, G0/G1 and S/G2 phases of the cell cycle were determined in naïve WT B cells and TazKD B cells and in these cells incubated with LPS for 48 h. The number of cells in Sub-G1, G0/G1 and S/G2 was unaltered in naïve TazKD B cells compared to naïve WT B cells (Fig. 1A). In contrast, LPS stimulation of TazKD B cells resulted in an increase of cells in G0/G1 (p=0.0142) and a decrease in S/G2 (p=0.0016) compared to WT B cells (Fig. 1B). We also found that stimulation of WT and TazKD B cells with LPS significantly decrease the percentage of cells in G0/G1 (p<0.001) and increase the cells in S/G2 (p<0.001) compared to naïve WT and naïve TazKD B cells (Fig. 1C). We confirmed that Taz protein was significantly reduced approximately 50% (p<0.001) in naïve B cells isolated from TazKD animals and that Taz protein remained reduced in LPS stimulated B cells (p<0.0001) (Fig. 1D). Activation of phosphoinositide 3-kinase (PI3K) is essential for B cell function and proliferation. Toll-like receptor-4 agonists such as LPS trigger PI3K activation and promote activation of the AKt signaling pathway [23]. PI3K, AKt, and pAKt protein expression were

examined in naïve and LPS stimulated WT and TazKD B cells. PI3K protein expression was decreased 40% (p=0.0229) in naïve TazKD B cells and 70% (p<0.0001) in LPS stimulated TazKD B cells compared to WT B cells, respectively (Fig. 1D). A greater reduction in PI3Kprotein expression was observed in LPS stimulated TazKD B cells compared to naïve TazKD B cells (p=0.0248, Fig. 1D). Basal AKt protein levels were unaltered in naïve and LPS stimulated TazKD B cells compared to naïve and LPS stimulated WT B cells, respectively. In contrast, pAKt protein levels were reduced 35% (p=0.0246) in naïve TazKD B cells and 80% (p<0.0001) in LPS stimulated TazKD B cells compared to naïve and LPS stimulated WT B cells, respectively (Fig. 1D). Although the ratio of pAKt/AKt protein was reduced minimally in naïve TazKD B cells compared to naïve WT B cells (p=0.0735), LPS stimulation resulted ina65%reduction (p=0.0002, Fig. 1D) in the ratio of pAKt/AKt protein in TazKD B cells compared to WT B cells. These data suggest that Taz-deficiency reduces proliferation and PI3K and AKt pathway activation in LPS activated B cells.

Proteomic analysis indicated alteration in the levels of several survival and apoptosis markers in LPS stimulated TazKD B cells compared to LPS stimulated WT B cells (Fig. 1E, Supplementary Figure 1, Supplementary Table 1). This included upregulation in the apoptotic markers Death-Inducer Obliterator 1, Beclin1, apoptosis regulator BAX, Caspase-6, Caspase-8, and Annexin A11. In addition, Aven, an anti-apoptotic protein that binds BCL-XL and impairs the activation of many Caspases [24], was down regulated in LPS stimulated TazKD B cells compared to WT B cells. In addition, the key cell cycle regulator cyclin-dependent kinase 9 [25] was down regulated in LPS stimulated TazKD B cells compared to WT B cells. Cyclin-dependent kinase 9 is serine/threonine kinase required for the formation of the catalytic core of the transcription elongation factor b and regulates the S phase of the cell cycle, proliferation, and differentiation of many cell types [26]. Transcription elongation factor b recruits RNA polymerase II and thus stimulates the transcription and elongation of many genes.

Similarly, the level of the cell cycle checkpoint protein HUS1,which negatively affects cell cycle control [27], was reduced in LPS stimulated TazKD B cells compared to WT B cells as was telomere length regulation protein 2. Telomere length regulation protein 2 is an essential protein for telomere length maintenance and thus prevents cellular senescence and favors the proliferation and survival of many cells [28]. Telomere length regulation protein 2 enhances the stability of phosphatidylinositol 3-kinase-related protein kinases. In addition, signal transducer and activator of transcription 1 was decreased in LPS stimulated TazKD B cells compared to WT cells. Signal transducer and activator of transcription 1 is a transcription factor necessary for the differentiation of naïve B cells into IgM antibody-producing marginal zone B cells thus ensuring a quick response to infectious pathogens and is essential for the transcription of many genes involved in immune cell division, survival, activation and recruitment [29]. These data suggest that Taz-deficiency impacts the survival and proliferation of LPS activated B lymphocytes.


A.
Naïve WT B cells
79.48%
G0/G1
7.19%
Sub-G1
7.70%
S/G2
Cell count
Fluorescence Intensity
Naïve TazKD B cells
77.69%
G0/G1
8.14%
Sub-G1
7.70%
S/G2
Cell count
Fluorescence Intensity
Naïve B cells
Percentage of cells
WT
TazKD
Sub-G1
G0/G1
S/G2
B.
LPS stimulated WT B cells
45.63%
G0/G1
33.89%
S/G2
10.39%
Sub-G1
Cell count
Fluorescence Intensity
LPS stimulated TazKD B cells
50.16%
G0/G1
31.65%
S/G2
12.66%
Sub-G1
Cell count
Fluorescence Intensity
LPS stimulated cells
Percentage of cells
WT
TazKD
Sub-G1
G0/G1
S/G2
C.
WT B cells
naïve cells
LPS stimulated cells
Percentage of cells
Sub-G1
G0/G1
S/G2
TazKD B cells
naïve cells
LPS stimulated cells
Percentage of cells
Sub-G1
G0/G1
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D.
Taz
Fold change
WT
TazKD
Naïve
LPS stimulated
PI3K
Fold change
WT
TazKD
Naïve
LPS stimulated
pAKt/AKt
Fold change
WT
TazKD
Naïve
LPS stimulated
Taz
Fold change
naïve
LPS
WT B cells
TazKD B cells
PI3K
Fold change
naïve
LPS
WT B cells
TazKD B cells
pAKt/AKt
Fold change
naïve
LPS
WT B cells
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Taz
PI3K
AKt
pAKt
β-actin

E.

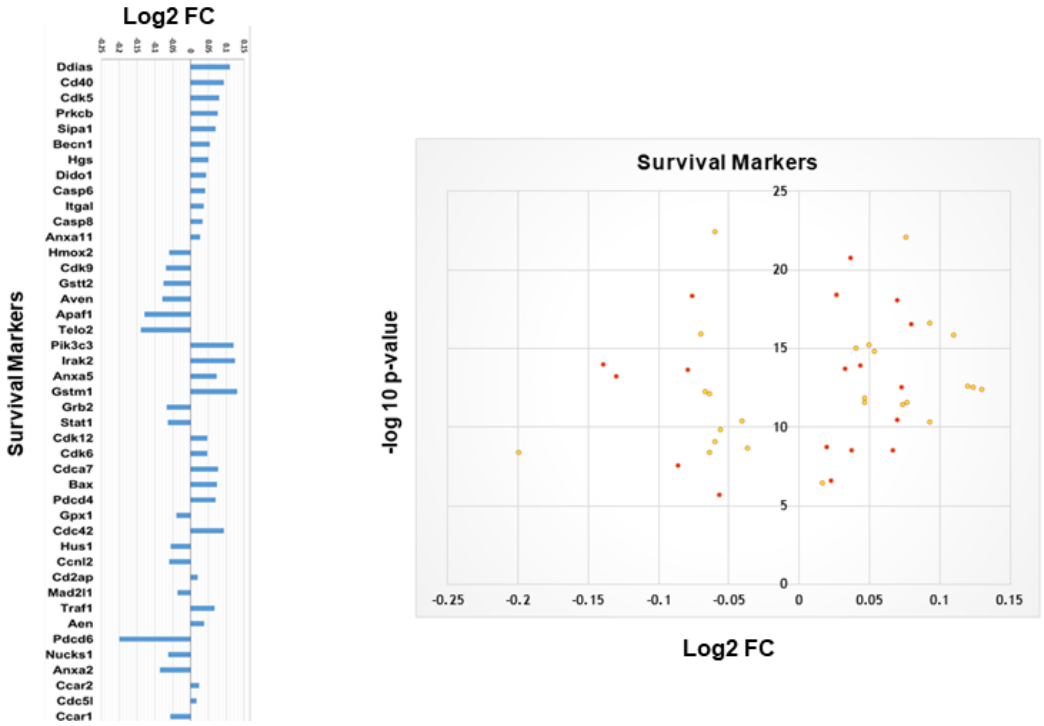


Figure 1. Taz-deficiency decreases cell proliferation and reduces cell survival markers in LPS stimulated B cells. The percentage of cells in Sub-G1, G0/G1 and S/G2 phases of the cell cycle and the protein levels of Taz, PI3K, AKt, and pAKt were determined in naïve WT and TazKD B cells or WT and TazKD B cells stimulated for 48 h with LPS as described in Materials and Methods. **A.** Flow cytometry representative figures of naïve WT and TazKD B cells and percentage of cells in Sub-G1, G0/G1 and S/G2, n=4-6. **B.** Flow cytometry representative figures of LPS stimulated WT and TazKD B cells and percentage of cells in Sub-G1, G0/G1 and S/G2, n=4-6. *p<0.05 compared with WT B cells. **C.** Comparing the percentage of cells in Sub-G1, G0/G1 and S/G2 in naïve WT vs LPS stimulated WT B cells and naïve TazKD vs LPS stimulated TazKD B cells separately. n=4-6. $^{\#}p$<0.05 compared with naïve cells. **D.** Protein levels of Taz, PI3K, AKt, and pAKt and β-Actin as loading control, n=4. *p<0.05 compared with WT B cells. **E.** Survival markers in LPS stimulated WT and TazKD B cells. Volcano plot depicting -log10 p-values vs log2 fold change (FC) and log2 fold change

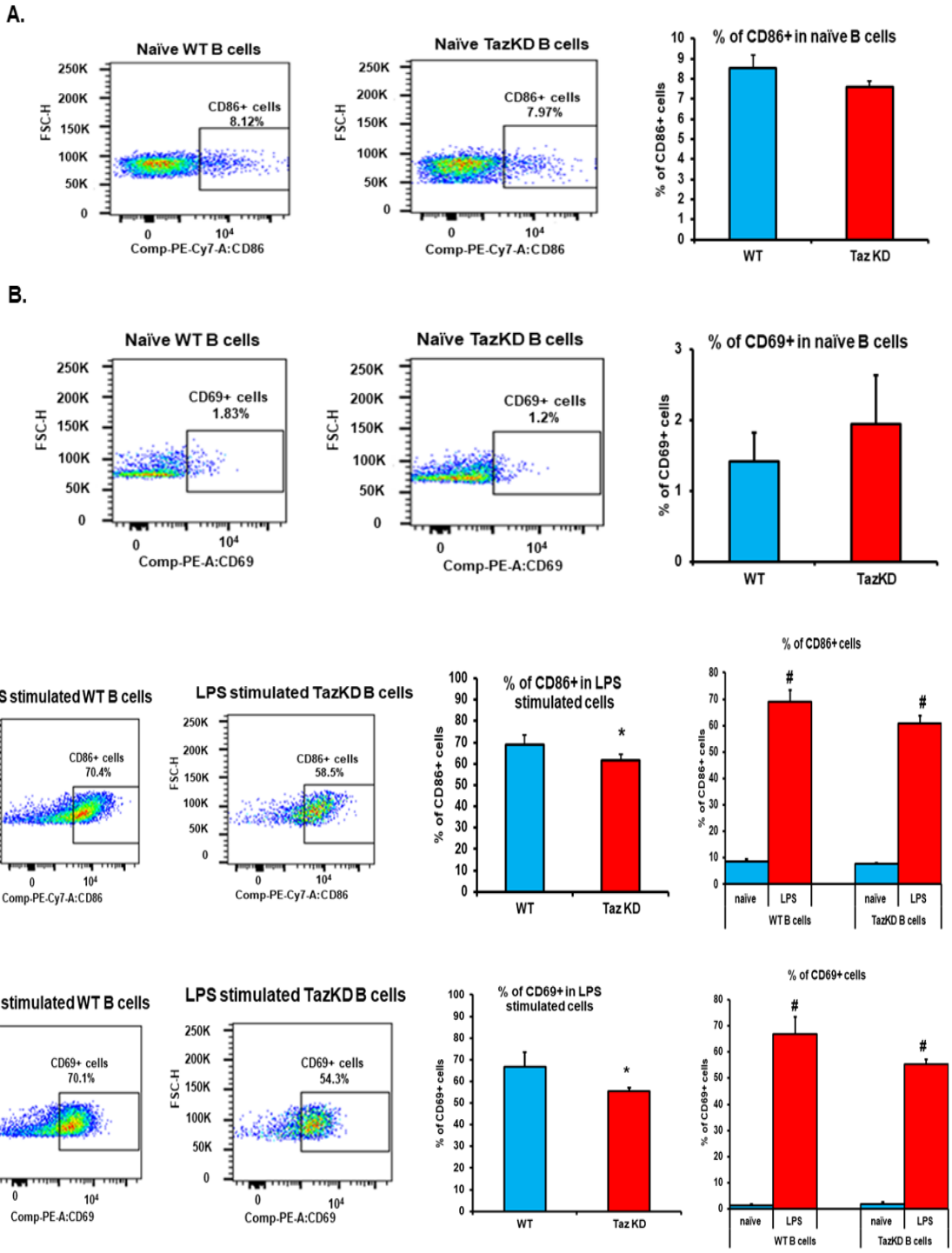
ratios of survival protein markers in LPS stimulated WT and TazKD B cells are shown. Significantly changed proteins are indicated in red. n=3, $p<0.05$ compared with WT B cells.

TAZ-deficiency reduces the expression of surface markers and immunogenic markers in LPS stimulated B cells

The above data indicated that Taz-deficiency impacts B cell proliferation. B lymphocytes are antigen-presenting cells and the ability of B cells to upregulate surface markers expression is essential for intercellular communication. Expression of the costimulatory molecule CD86 in B cells is required to initiate interaction with T lymphocytes [30]. In addition, activated B cells exhibit CD69 induction, a marker of early leukocyte activation [31]. We measured CD86 and CD69 expression on the surface of naïve and LPS stimulated WT and TazKD B cells. As expected, minimal expression of CD86 and CD69 was observed in both naïve WT and TazKD B cells and Taz deficiency did not impact their expression (Figs. 2A, 2B). In contrast, CD86 and CD69 expression were markedly upregulated in both WT B cells and TazKD B cells stimulated with LPS (Figs. 2C, 2D). However, we observed a10% reduction (p=0.0172) in CD86 expression and an 15% reduction (p=0.0027) in CD69 expression in LPS stimulated TazKD B cells compared to LPS stimulated WT B cells (Figs. 2C, 2D).

Proteomic analysis indicated alteration in the level of several other B lymphocyte related receptors and markers in stimulated TazKD B cells compared to WT B cells (Fig. 2E, Supplementary Figure 1, Supplementary Table 2). For example, lymphocyte-specific protein 1, a cytoskeletal protein expressed in B lymphocytes and other immune cells required for the migration of these cells to the site of inflammation [32], was reduced in LPS stimulated TazKD B cells compared to WT B cells. Immunity-related GTPase family M protein 1, a member of a family of interferon-inducible cytoplasmic immunity-related GTPases required for mitochondrial homeostasis in B lymphocytes [33], was reduced in LPS stimulated TazKD B cells compared to WT B cells. Deficiency in immunity-related GTPase family M protein 1 is known to weaken the adaptive immune system and phagosome maturation and was reduced in

LPS stimulated TazKD B cells compared to WT B cells. In contrast, the level of B- and T-lymphocyte attenuator was increased in LPS stimulated TazKD B cells compared to WT B cells. B- and T-lymphocyte attenuator is an “off” signal that inhibits the maturation of B-lymphocytes and is expressed in high amounts in naïve and immature B-lymphocytes in comparison with active and mature B-lymphocytes [34]. In addition, the level of cluster of differentiation-22 was increased in LPS stimulated TazKD B cells compared to WT B cells. Cluster of differentiation-22 confines B cell differentiation to plasmablasts, inhibits interleukin-6 production and increases interleukin-10 production, thus inducing an immune-tolerance state [35, 36]. Finally, pro-interleukin-16 was increased in LPS stimulated TazKD B cells compared to WT B cells. Pro-interleukin-16 is a mediator of apoptosis through activation of caspase-3 in B lymphocytes [37]. These data suggest that Taz-deficiency alters expression of B lymphocyte receptors and immunogenic markers.


A.
Naïve WT B cells
CD86+ cells
8.12%
FSC-H
Comp-PE-Cy7-A:CD86
Naïve TazKD B cells
CD86+ cells
7.97%
% of CD86+ in naïve B cells
% of CD86+ cells
WT
Taz KD
B.
Naïve WT B cells
CD69+ cells
1.83%
Comp-PE-A:CD69
Naïve TazKD B cells
CD69+ cells
1.2%
% of CD69+ in naïve B cells
% of CD69+ cells
WT
TazKD
C.
LPS stimulated WT B cells
CD86+ cells
70.4%
LPS stimulated TazKD B cells
CD86+ cells
58.5%
% of CD86+ in LPS stimulated cells
% of CD86+ cells
WT
Taz KD
% of CD86+ cells
naïve
LPS
WT B cells
TazKD B cells
D.
LPS stimulated WT B cells
CD69+ cells
70.1%
LPS stimulated TazKD B cells
CD69+ cells
54.3%
% of CD69+ in LPS stimulated cells
% of CD69+ cells
WT
Taz KD
% of CD69+ cells
naïve
LPS
WT B cells
TazKD B cells

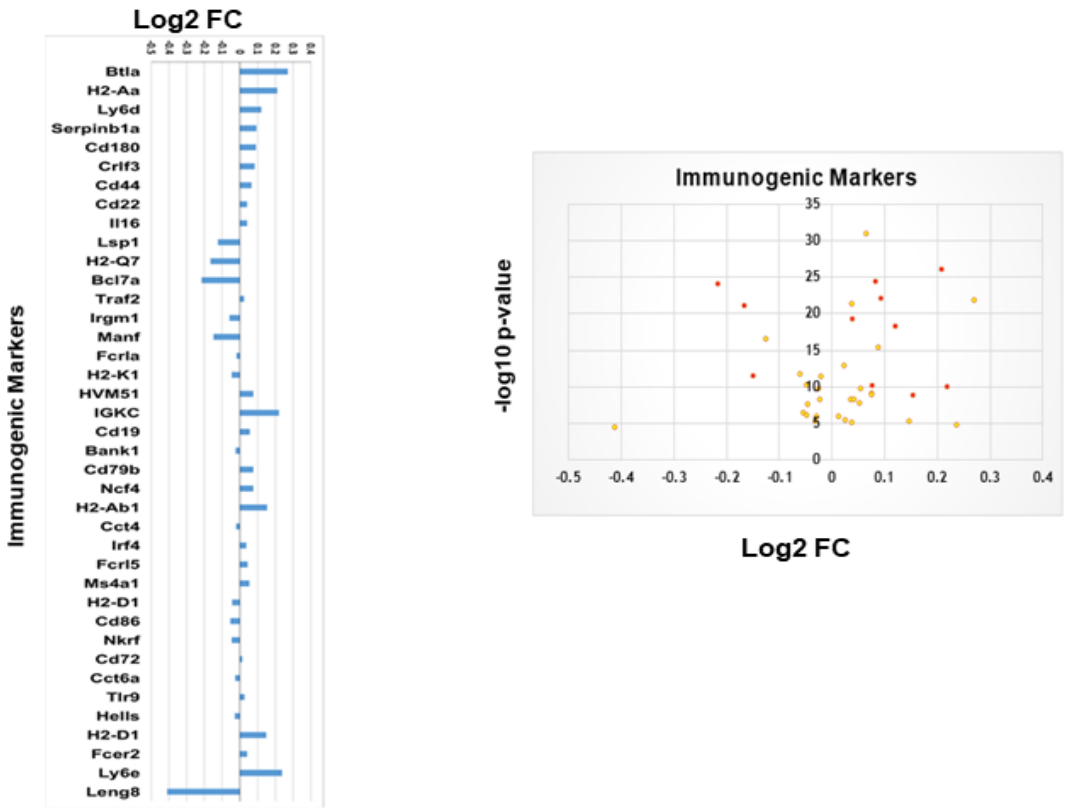


Figure 2. Taz-deficiency reduces the expression of B cell surface markers and immunogenic markers. The percent of CD86+ and CD69+ expressing cells was determined in naïve WT and TazKD B cells or WT and TazKD B cells stimulated for 48 h with LPS as described in Materials and Methods. Representative flow cytometry histograms are shown in A-D. **A.** CD86+in naïve WT and TazKD B cells. **B.** CD69 expression in naïve WT and TazKD B cells. **C.** CD86+ expression in LPS stimulated WT and TazKD B cells. **D.** CD69+ expression in LPS stimulated WT and TazKD B cells. n=3, *p<0.05 compared with WT B cells and $^{\#}p$<0.05 compared with naïve cells. **E.** Immunogenic markers in LPS stimulated WT and TazKD B cells. Volcano plot depicting -log10 p-values vs log2 fold change (FC) and log2 fold change ratios of immunogenic markers in LPS stimulated WT and TazKD B cells is shown. Significantly changed proteins are indicated in red. n=3, p<0.05 compared with WT B cells.

TAZ-deficiency reduces secretion of chemokines and IgM in LPS stimulated B cells

Activated B cells synthesize and secret antibodies. Cells from WT and TazKD mice were isolated and stimulated with LPS for 72 h the supernatants collected and level of the secreted antibodies IgG and IgM determined. The level of IgG in the supernatant was unaltered in LPS stimulated TazKD B cells compared to WT B cells (Fig. 3A). In contrast, the level of IgM was reduced 20% (p<0.0001) in the supernatant of LPS stimulated TazKD B cells compared to WT B cells (Fig. 3B). Activated B cells also secrete chemokines that promote leukocyte recruitment to sites of infections in order to increase the immune response. Murine B cells synthesize and secrete two CXC chemokines, cytokine-induced neutrophil chemoattractant (KC) and macrophage inflammatory protein-2 (MIP-2) in response to LPS [4]. B cells from WT and TazKD mice were isolated and stimulated with LPS for 24 h the supernatants collected and level of secreted KC and MIP-2 determined. The level of KC was decreased 55% (p<0.0001) and the level of MIP-2 was decreased 50% (p<0.0001) in the supernatant of LPS stimulated TazKD B cells compared to WT B cells (Fig. 3C, 3D). Thus, Taz-deficiency reduces secretion of KC and MIP-2 chemokines and IgM in LPS stimulated B cells.

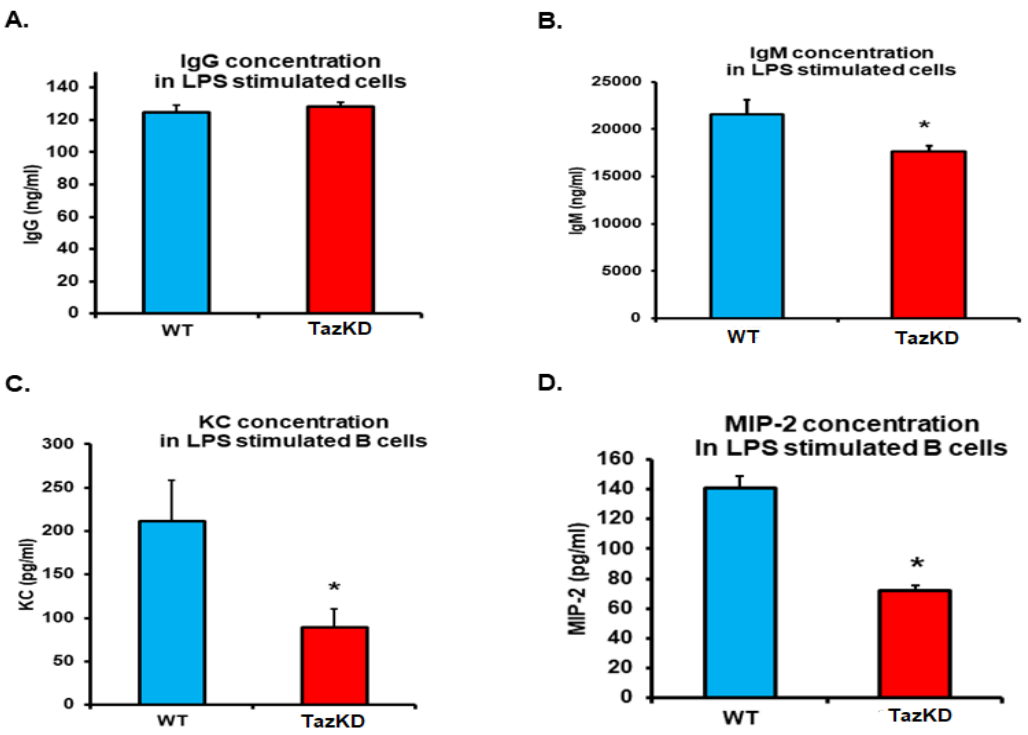


Figure 3. Taz-deficiency reduces secretion of chemokines and IgM in LPS stimulated B cells. B cells were isolated from WT or TazKD mice and stimulated with LPS for 72 h for measurement of antibodies concentrations or stimulated for 24 h with LPS for measurement of chemokines concentrations as described in Materials and Methods. **A.** IgG. **B.** IgM. **C.** KC. **D.** MIP-2. n=4-6, *p<0.05 compared with WT B cells.

TAZ-deficiency reduces proteasome and immunoproteasome activities in LPS stimulated B cells

The ubiquitin proteasome system is the main cellular protein degradation pathway which degrades damaged proteins by proteolysis and it is an ATP-consuming process [38]. Inactivation of the 26S proteasome inhibits activation and function of many immune cells including B lymphocytes [39, 40]. Proteasome activity was determined in naïve B cells from WT and TazKD mice or WT and TazKD B cells stimulated with LPS for 24 h. Proteasome activity was reduced approximately 15% ($p<0.011$) in naïve TazKD B cells compared to naïve WT B cells (Fig. 4A). In addition, proteasome activity was decreased approximately 60% ($p<0.0001$) in LPS stimulated TazKD B cells compared to LPS stimulated WT B cells (Fig. 4B). The reduction in proteasome activity was greater in LPS stimulated TazKD B cells compared to naïve TazKD B cells (Fig. 4A, B).

The immunoproteasome is a specialized form of proteasome that is crucial for antigen presentation performed by various activated immune cells [41]. Immunoproteasome activity was determined in naïve B cells from WT and TazKD mice or WT and TazKD B cells stimulated with LPS for 24 h. Immunoproteasome activity was unaltered in naïve TazKD B cells compared to naïve WT B cells (Fig. 4C). In contrast, immunoproteasome activity was reduced 25% ($p<0.0001$) in LPS stimulated TazKD B cells compared to LPS stimulated WT B cells (Fig. 4D). We investigated that LPS stimulation potentially increased the proteasome and immunoproteasome activities in WT stimulated cells compared to naïve WT B cells ($p<0.0001$, Fig. 4E and 4F). Also, we found that the proteasome and immunoproteasome activities were significantly increased in TazKD stimulated cells compared to naïve TazKD B cells ($p<0.0001$, Fig. 4E and 4F).

Proteomic analysis indicated alteration in the level of several B lymphocyte proteasomal proteins in LPS stimulated TazKD B cells compared to LPS stimulated WT B

cells (Fig. 4G, Supplementary Figure 1, Supplementary Table 3). These included a reduction in proteasome activator complex subunit 1 and the induced proteolytic core subunit proteasome subunit beta type-10 which are linked to reduced function of the immunoproteasome and thus a defect in antigen presentation [42]. Ubiquitin carboxyl-terminal hydrolase 48 promotes survival of cells by stabilizing their genetic content [43]and was reduced in LPS stimulated TazKD B cells compared to WT B cells. The 26S proteasome recognizes misfolded proteins or proteins assigned for degradation by tagging these proteins with ubiquitin proteins [44]. This ubiquitination requires the combined function of ubiquitin activating enzymes E1,ubiquitin conjugating enzymes E2, and E3 ligases [45]. Several ubiquitination enzymes exhibited reduced level of expression in LPS stimulated TazKD B cells compared to LPS stimulated WT B cells. These included the conjugating enzymes ubiquitin-conjugating enzyme E2 variant-1, -2, and ubiquitin-conjugating enzyme E2 L3, and the E3 ligase ring finger protein 213. In addition, many 19S proteasome subunits, deubiquitinases and ubiquitin receptors required for binding ubiquitinated tagged proteins [46] were decreased in LPS stimulated TazKD B-lymphocytes compared to LPS simulated WT B cells including26S proteasome non-ATPase regulatory subunit 8, 26S proteasome non-ATPase regulatory subunit 14, and 26S proteasome non-ATPase regulatory subunit 10. These observed decreases in ubiquitin-26S proteasome related proteins coincided with the reduced proteasomal and immunoproteasome activities in LPS stimulated TazKD B cells. Thus, Taz-deficiency reduces proteasomal and immunoproteasome activities in LPS stimulated B cells.

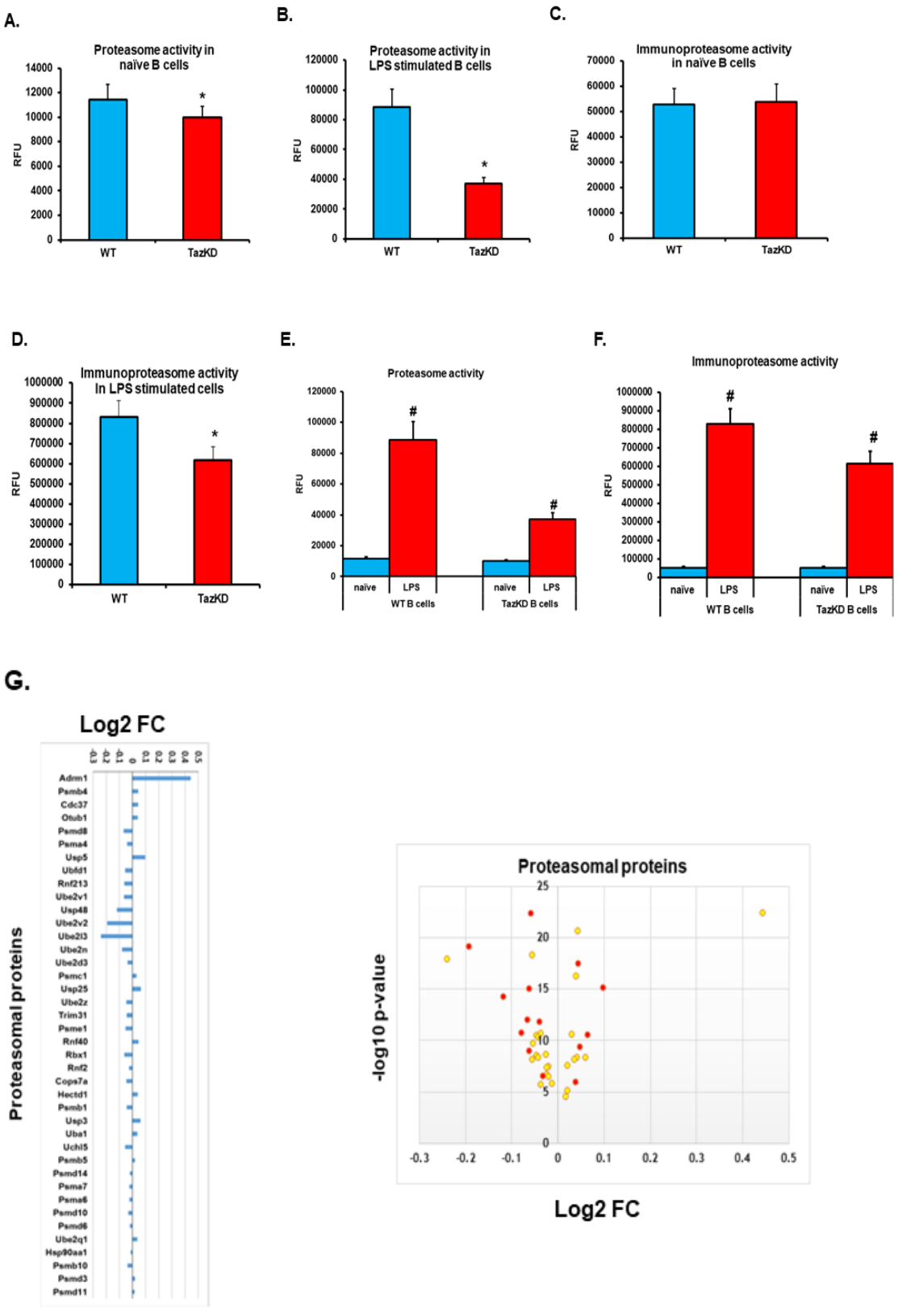


Figure 4. TAZ-deficiency reduces proteasome and immunoproteasome activity in LPS stimulated B cells. Proteasome and immunoproteasome activities were determined in naïve B cells from WT and TazKD mice or WT and TazKD B cells stimulated with LPS for 24 h as

described in Materials and Methods. **A.** Proteasome activity in naïve WT and TazKD B cells. **B.** Proteasome activity in LPS stimulated WT and TazKD B cells. **C.** Immunoproteasome activity in naïve WT and TazKD B cells. **D.** Immunoproteasome activity in LPS stimulated WT and TazKD B cells. **E.** Proteasome activity in naïve WT vs LPS stimulated WT B cells and naïve TazKD vs LPS stimulated TazKD B cells separately. **F.** Immunoproteasome activity in naïve WT vs LPS stimulated WT B cells and naïve TazKD vs LPS stimulated TazKD B cells separately. n=4, $*p<0.05$ compared with WT B cells and $^{\#}p<0.05$ compared with naïve cells. **G.** Proteasomal proteins in LPS stimulated WT and TazKD B cells. Volcano plot depicting -log10 p-values vs log2 fold change (FC) and log2 fold change ratios of proteasomal proteins in LPS stimulated WT and TazKD B cells are shown. Significantly changed proteins are indicated in red. n=3, $p<0.05$ compared with WT B cells.

TAZ-deficiency reduces metabolic activity in LPS stimulated B cells

Taz gene mutations are associated with mitochondrial dysfunction in BTHS patients. It has been reported that the stability of mitochondrial supercomplexes is essential for proper mitochondrial activity and any disruption in these complexes results in mitochondrial dysfunction like that observed in BTHS patients [10]. We measured the protein expression of five mitochondrial complexes in LPS stimulated WT and TazKD B cells. We found complex IV protein level was significantly reduced 40% ($p<0.001$) in TazKD B cells compared to WT B cells. Complex IV deficiency was observed in BTHS models and lymphoblasts from BTHS patients (Supplementary Figure 2). We also measured Cytochrome c oxidase subunit I (MTCO1) expression in LPS stimulated WT and TazKD B cells. MTCO1 is one of mitochondrial DNA (mtDNA) encoded subunits of Complex IV. We observed that MTCO1 protein level was significantly decreased 50% ($p<0.001$) in TazKD B cells compared to WT B cells (Fig. 5A). Brandner et al reported that deletion of Taz gene in yeast (Taz1) leads to instability of mitochondrial supercomplexes by affecting Complex IV stability [47]. In addition, primary lymphoblasts from BTHS patients showed dissociation of complex IV which results in destabilization of respiratory chain supercomplexes and mitochondrial dysfunction [17].

Naïve B cells are metabolically quiescent whereas LPS activation of B cells initiates a metabolic reprogramming which includes the augmentation of mitochondrial oxidative phosphorylation (OXPHOS) required for B cell proliferation and function [3]. Oxygen consumption rates (OCR) were determined in WT B cells and TazKD B cells after stimulation with LPS for 48 h using a Seahorse XF24 analyzer. Basal respiration (measured as OCR before adding inhibitors) was reduced 25% ($p<0.0001$) in LPS stimulated TazKD B cells compared to WT B cells (Fig. 5B). Maximal respiration (measured as OCR after adding FCCP an uncoupler of the electron transport chain) was approximately 25% lower ($p<0.0001$) in LPS

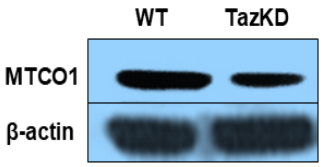
stimulated TazKD B cells compared to WT B cells (Fig. 5B). In addition, spare respiratory capacity (calculated by the difference between the maximal OCR and the basal OCR) was reduced 35% ($p<0.0001$) in LPS stimulated TazKD B cells compared to WT B cells (Fig. 5B).

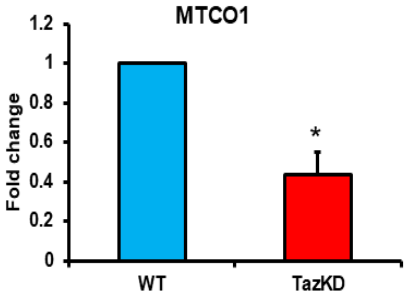
Previous studies have demonstrated that naïve B cells increase glycolysis upon LPS stimulation [48]. Glycolytic activity was determined in WT B cells and TazKD B cells after stimulation with LPS for 48h using a Seahorse XF24 analyzer. Glycolysis (measured by determination of the change in extracellular acidification rate (ECAR) before oligomycin addition minus ECAR before glucose addition) was reduced 30% ($p<0.0001$) in LPS stimulated TazKD B cells compared to WT B cells (Fig. 5C). Glycolytic capacity (calculated as the difference between ECAR following addition of oligomycin minus basal ECAR), was reduced 50% ($p<0.0001$) in LPS stimulated TazKD B cells compared to WT B cells (Fig. 5C). Finally, glycolytic reserve (calculated as the difference between glycolytic capacity and glycolysis) was decreased 75% ($p<0.0001$) in LPS stimulated TazKD B cells compared to WT B cells (Fig. 5C). Thus, TAZ-deficiency reduces metabolic activity in LPS stimulated B cells.

Proteomic analysis indicated significant alteration in the level of many B lymphocyte mitochondrial proteins in LPS stimulated TazKD B cells compared to WT B cells (Fig. 5D, Supplementary Figure 1, Supplementary Table 4). Among these included reduction in NADH dehydrogenase (ubiquinone) flavoprotein 1& 2 core subunits in mitochondrial Complex1, the first of five mitochondrial complexes involved in the oxidative phosphorylation cascade [49]. Cytochrome c oxidase assembly factor 7 is one of the 14 assembly subunits required for the formation of functional cytochrome c oxidase Complex IV [50]. Cytochrome c oxidase assembly factor 7 levels were lower in LPS stimulated TazKD B cells compared to WT B cells. Translocase of the outer membrane 7, an essential assembly subunit for the translocase of the outer membrane complexes and translocase of the outer membrane 20 and translocase of the outer membrane 22 [51], were reduced in LPS stimulated TazKD B cells compared to WT B

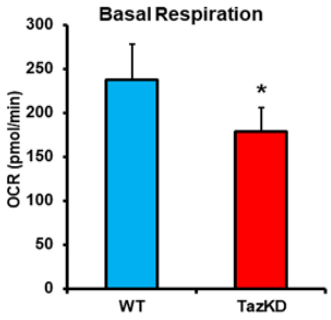
cells. The level of mitochondrial import inner membrane translocase subunit TIM16, which forms a complex with mitochondrial import inner membrane translocase subunit TIM14 to facilitate its insertion via hydrophobic N-terminal domain with the presequence translocases of the inner mitochondrial membrane [52], was reduced in LPS stimulated TazKD B cells compared to WT B cells. Finally, the level of cytochrome c, which is a main participant in the electron transport chain of mitochondria, and ATP synthase required for ATP production in oxidative phosphorylation (ATP synthase F1 subunit beta), were reduced in LPS stimulated. TazKD B cells compared to WT B cells. Thus, Taz-deficiency reduces mitochondrial function in LPS stimulated TazKD B lymphocytes.

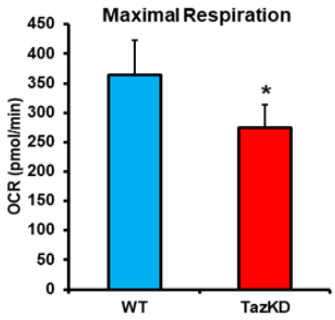
A.

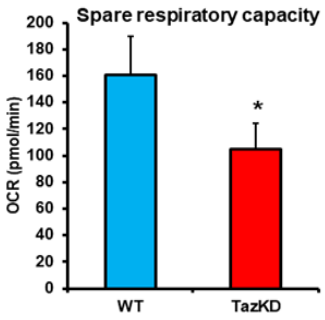

WT
TazKD
MTCO1
β-actin


MTCO1
Fold change
1.2
1
0.8
0.6
0.4
0.2
0
*
WT
TazKD

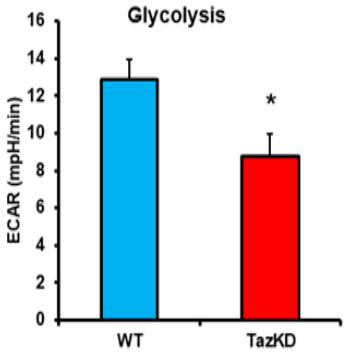
B.

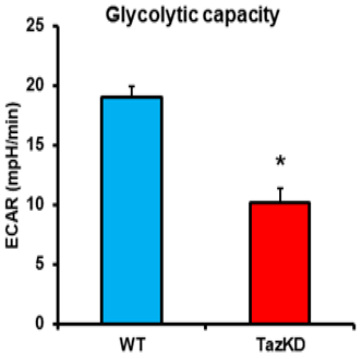

Basal Respiration
OCR (pmol/min)
300
250
200
150
100
50
0
*
WT
TazKD

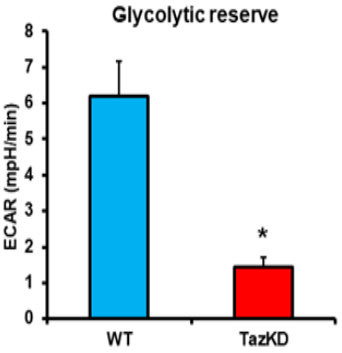

Maximal Respiration
OCR (pmol/min)
450
400
350
300
250
200
150
100
50
0
*
WT
TazKD


Spare respiratory capacity
OCR (pmol/min)
200
180
160
140
120
100
80
60
40
20
0
*
WT
TazKD

C.


Glycolysis
ECAR (mpH/min)
16
14
12
10
8
6
4
2
0
*
WT
TazKD


Glycolytic capacity
ECAR (mpH/min)
25
20
15
10
5
0
*
WT
TazKD


Glycolytic reserve
ECAR (mpH/min)
8
7
6
5
4
3
2
1
0
*
WT
TazKD

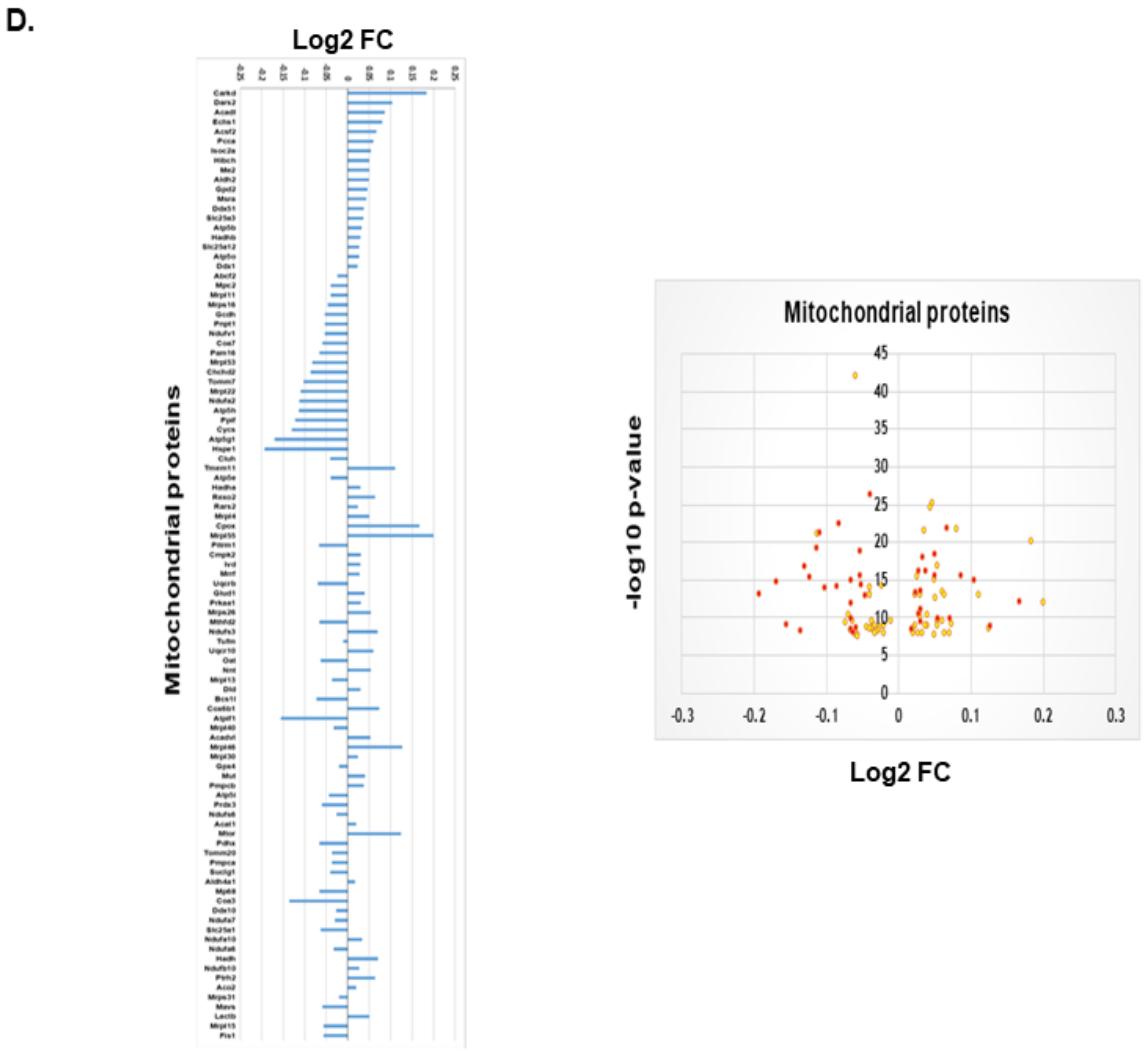


Figure 5. Taz-deficiency reduces metabolic activity in LPS stimulated B cells. Mitochondrial function and glycolytic activity were analyzed in LPS stimulated WT and TazKD B cells using a Seahorse XF24 analyzer as described in Materials and Methods. **A.** MTCO1 protein level in LPS stimulated WT and TazKD B cells. **B.** Basal respiration, maximal respiration and spare respiratory capacity. **C.** Glycolysis, glycolytic capacity and glycolytic reserve. n=3, **p*<0.05 compared with WT B cells. **D.** Mitochondrial proteins in LPS stimulated WT and TazKD B cells. Volcano plot depicting -log10 p-values vs log2 fold change (FC) and log2 fold change ratios of mitochondrial proteins in LPS stimulated WT and TazKD B cells are shown. Significantly changed proteins are indicated in red n=3, *p*<0.05 compared with WT B cells.

4.5 Discussion

B lymphocytes are responsible for humoral immunity and play a key role in the immune response and optimal mitochondrial function is required to support activated B cell function[2]. In this study, we examined how deficiency of Taz, a cardiolipin remodeling enzyme required for mitochondrial function, alters the metabolic activity of B cells and their response to activation by LPS in mice. We have demonstrated for the first time that Taz-deficiency in LPS activated mouse B cells results in impaired mitochondrial function and this may contribute to reduced B cell function.

Mitochondria play a vital role in the activation, survival, development, and differentiation of B lymphocytes and their response to various phenotypic and environmental changes [53]. Mitochondria are a highly dynamic organelle that relies on many proteins and enzymes to maintain their membrane composition, membrane potential, and control continuous rounds of fusion and fission in order to remain fully competent for respiration and ATP synthesis [54]. Naïve B cells predominately exist in a quiescent state until stimulation [53]. Activated B cells are metabolically reprogrammed resulting in an increase in both glycolysis and OXPHOS pathways required for rapid proliferation and differentiation [3]. It is well established that TAFAZZIN mutation is associated with mitochondrial dysfunction in different organs of BTHS patients including the heart and skeletal muscle [10]. Moreover, multiple studies have reported that Taz-deficiency in TazKD mice result in decreased mitochondrial respiration in several tissues [55, 56]. We previously reported that Epstein-Barr virus transformed human BTHS lymphoblasts exhibited impairment in mitochondrial function [57]. Herein, we demonstrate for the first time that LPS stimulated B cells isolated from TazKD mice exhibit a marked impairment in OXPHOS. In addition, our proteomic analysis identified a decrease in several mitochondrial proteins including NADH dehydrogenase (ubiquinone) flavoprotein 1& 2 core subunits in mitochondrial Complex1, cytochrome c oxidase assembly

factor 7, translocase of the outer membrane 7, import inner membrane translocase subunit TIM16, cytochrome c, and ATP synthase in LPS stimulated TazKD B cells compared to LPS stimulated WT B cells. Collectively, a reduction in the above mitochondrial proteins and impairment in OXPHOS indicate a marked defect in mitochondrial function of LPS activated B cells from TazKD mice.

Activation of B cells with LPS increases cell proliferation, induces surface marker expression, chemokine signaling, and promotes B cell differentiation into antibody secreting cells required to amplify the immune response [3, 4]. PI3K activation is essential for B cell proliferation and is initiated upon B cell stimulation. The AKt serine/threonine kinase is one of the critical signaling molecules of the PI3K activation pathway. Increasing evidence suggests that reduced activity of the PI3K pathway and insufficient AKt activation inhibits glucose utilization in B cells [23]. For example, inhibition of PI3K activity in WT B cells by LY294002 treatment was shown to diminish glycolysis [58]. Consistent with attenuation of PI3K activity in LPS stimulated TazKD B cells, we observed impaired glycolytic activity. In B cells, glycolysis is crucial for their proliferation and antibody production and inhibiting glycolysis leads to impaired B cell growth and suppresses antibody secretion [3].Our observation of a reduced number of cells entering the S/G2 phase of the cell cycle and reduced IgM secretion in LPS stimulated TazKD B cells is consistent with these results. In addition, we observed a reduction in CD69 and CD86 surface marker expression in LPS stimulated TazKD B cells which may potentially result in reduced intercellular communication with other cells of the immune system. MIP-2 and KC, CXC family member chemokines, are secreted by activated murine B cells and are known to recruit neutrophils to infection sites [4]. B cells isolated from TazKD mice exhibited a reduced ability to produce MIP-2 and KC after LPS activation. Thus, decreased MIP-2 and CK secretion by TazKD B cells could potentially inhibit neutrophil recruitment and increase susceptibility to infection. Together, these results indicate that LPS

activated B cells from TazKD mice exhibit a reduced function in their ability to amplify the immune response.

The ubiquitin proteasome system is the main protein degradation pathway which degrades damaged proteins by proteolysis [59]. The 26S proteasome is a multiprotein complex and is considered the master protein degradation machinery in cells [59]. The 26S proteasome degradation machinery regulates many cellular processes including proliferation, DNA repair, cell cycle, antigen presentation, and autophagy. The 26S proteasome consists of two large complexes, the 19S proteasome or the regulatory complex and the 20S proteasome where the proteolytic core is localized. Intact function of the 26S proteasome requires strong binding between the 19S proteasome and 20S proteasome and this assembly is crucial for optimal degradation function of the 26S proteasome complex [60]. Many autoimmune diseases, neurodegenerative disorders and cancers can be linked to alteration in 26S proteasome assembly and function. It was previously reported that inactivation of the 26S proteasome inhibits the function of many immune cells including B lymphocytes resulting in their apoptosis [40]. Moreover, mitochondrial dysfunction is induced by defective 26S proteasome machinery [61]. In addition, efficient and intact 26S proteasome activity is required to remove damaged proteins from the mitochondria and maintain mitochondrial homeostasis [55,56]. The immunoproteasome is a specialized form of proteasome crucial for the antigen presentation elicited by different immune cells [63]. It is constitutively expressed in immune cells especially in antigen-presenting cells such as B lymphocytes. The immunoproteasome is composed of a cap known as the proteasome activator complex subunit 1 or 11Sproteasome and an induced form of the 20S proteasome that exhibits a specialized proteolytic core for antigenic proteins [59]. It was previously demonstrated that immunoproteasome inhibition impairs naïve B cell activation [41]. Consistent with reduced proteasome and immunoproteasome activities, our proteomic data revealed for the first time that LPS stimulated TazKD B cells exhibited a

significant reduction in the level of 26S proteasome subunits and chaperones. The observed alteration in proteasome subunits might contribute to the mitochondrial dysfunction in LPS stimulated TazKD B cells. In addition, several 19S proteasome subunits, deubiquitinases and ubiquitin receptors required for binding the ubiquitinated tagged proteins were decreased in LPS stimulated TazKD B cells. A decrease in these proteasome related proteins in LPS stimulated TazKD B cells may additionally contribute to reduced activity of the proteasome machinery and survival of these cells.

Historically regarded as a cardiac disease, BTHS is now considered a multi-system disorder [9]. In addition to the multitude of clinical features, BTHS boys exhibit an increased susceptibility to infections attributed to neutropenia [8, 9]. The neutropenia in BTHS is treated with granulocyte colony stimulating factor (G-CSF) [64]. G-CSF works by stimulating the bone marrow to form and maturate neutrophils. The clinical data, routine blood counts, and responses to G-CSF therapy from 88 affected boys was recently reviewed [65]. It was concluded that susceptibility to infections is due only in part to neutropenia since in some instances infection may occur despite consistent prevention of neutropenia by G-CSF therapy. This suggests the possibility that defective function in other immune cell types may contribute to infections in BTHS. Defective B lymphocyte function might contribute to an overall immune system dysfunction through reduced intercellular communication and antigen recognition and further increase the susceptibility of BTHS patients to infections. For example, naïve B cells, when activated, secrete antibodies, cytokines and chemokines that amplify the immune response to pathogens and promote the recruitment of leukocytes to sites of infections. Activated murine B cells synthesize and secrete CXCL1 (KC) and CXCL2 (MIP-2), functional homologues of human CXCL8 (IL-8) and GRO-chemokines [4, 66]. These CXC family chemokines recruit neutrophils to sites of infection [4, 66]. Thus, defective B cell function in

BTHS could also result in reduced ability of neutrophils, with normal killing/phagocytosis ability [11], to ward off infections if not properly directed to sites of infections.

In conclusion, we have demonstrated for the first time that LPS activated TazKD B cells exhibit reduced mitochondrial OXPHOS and glycolysis, reduced proliferation, lowered expression of CD86 and CD69 surface markers, reduced secretion of IgM antibody and the KC and MIP-2 chemokines, and reduced proteasome and immunoproteasome activities. In addition, our proteomic analysis revealed striking alterations in protein targets that regulate cell survival, immunogenicity, proteasomal processing and mitochondrial function consistent with these functional observations. These studies highlight a novel role for Taz in the maintenance of normal B cell function.

Conflict of interest statement

The authors of this study have no conflict of interest.

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Author contributions

H. Zegallai, E. Abu-El-Rub, E. Mejia, J. Field and L. Cole performed all experiments and analyzed the data. J. Gordon provided reagents and analytic tools. A. Marshall, H. Zegallai and

G. Hatch were responsible for conceptual design. H. Zegallai and G. Hatch wrote the manuscript. All authors edited the manuscript.

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Supplementary Table 1. Survival proteins in LPS stimulated B cells

Protein symbol	Protein name	Fold change	P-value
Ddias	DNA damage-induced apoptosis suppressor protein	0.1103	0.0261
Cd40	Tumor necrosis factor receptor superfamily member 5	0.0935	0.0219
Cdk5	Cyclin-dependent-like kinase 5	0.0805	0.0221
Prkcb	Protein kinase C beta type	0.0764	0.0062
Sipa1	Signal-induced proliferation-associated protein 1	0.0700	0.0156
Becn1	Beclin-1	0.0537	0.0332
Hgs	Hepatocyte growth factor-regulated tyrosine kinase substrate	0.0502	0.0299
Dido1	Death-inducer obliterator 1	0.0436	0.0408
Casp6	Caspase-6	0.0407	0.0314
Itgal	Integrin alpha-L	0.0368	0.0084
Casp8	Caspase-8	0.0333	0.0424
Anxa11	Annexin A11	0.0265	0.0145
Hmox2	Heme oxygenase 2	-0.0598	0.0058
Cdk9	Cyclin-dependent kinase 9	-0.0692	0.0257
Gstt2	Glutathione S-transferase theta-2	-0.0761	0.0148
Aven	Cell death regulator Aven	-0.0792	0.0436
Apaf1	Apoptotic protease-activating factor 1	-0.1295	0.0474
Telo2	Telomere length regulation protein TEL2 homolog	-0.1391	0.0399
Pik3c3	Phosphatidylinositol 3-kinase catalytic subunit type 3	0.1203	0.0552
Irak2	Interleukin-1 receptor-associated kinase-like 2	0.1239	0.0556
Anxa5	Annexin A5	0.0733	0.0564
Gstm1	Glutathione S-transferase Mu 1	0.1301	0.0581
Grb2	Growth factor receptor-bound protein 2	-0.0669	0.0593
Stat1	Signal transducer and activator of transcription 1	-0.0636	0.0615
Cdk12	Cyclin-dependent kinase 12	0.0471	0.0658
Cdk6	Cyclin-dependent kinase 6	0.0470	0.0696
Cdca7	Cell division cycle-associated protein 7	0.0772	0.0698
Bax	Apoptosis regulator BAX	0.0738	0.0720
Pdcd4	Programmed cell death protein 4	0.0702	0.0905
Gpx1	Glutathione peroxidase 1	-0.0400	0.0910
Cdc42	Cell division control protein 42 homolog	0.0934	0.0924
Hus1	Checkpoint protein HUS1	-0.0560	0.1049
Ccnl2	Cyclin-L2	-0.0594	0.1249
Cd2ap	CD2-associated protein	0.0201	0.1352
Mad2l1	Mitotic spindle assembly checkpoint protein MAD2A	-0.0362	0.1357
Traf1	TNF receptor-associated factor 1	0.0670	0.1400
Aen	Apoptosis-enhancing nuclease	0.0374	0.1413
Pdcd6	Programmed cell death protein 6	-0.1993	0.1444
Nucks1	Nuclear ubiquitous casein and cyclin-dependent kinase substrate1	-0.0633	0.1451

Anxa2	Annexin A2	-0.0861	0.1748
Ccar2	Cell cycle and apoptosis regulator protein 2	0.0233	0.2196
Cdc5l	Cell division cycle 5-like protein	0.0165	0.2261
Ccar1	Cell division cycle and apoptosis regulator protein 1	-0.0568	0.2715

Supplementary Table 2. Immunogenic proteins in LPS stimulated B cells

Protein symbol	Protein name	Fold change	P-value
Btla	B- and T-lymphocyte attenuator	0.271	0.007
H2-Aa	H-2 class II histocompatibility antigen, A-B alpha chain	0.210	0.002
Ly6d	Lymphocyte antigen 6D	0.120	0.015
Serpinb	Leukocyte elastase inhibitor A	0.093	0.006
Cd180	CD180 antigen	0.090	0.030
Crlf3	Cytokine receptor-like factor 3	0.083	0.004
Cd44	CD44 antigen	0.067	0.001
Cd22	B-cell receptor CD22	0.040	0.008
Il16	Pro-interleukin-16	0.040	0.012
Lsp1	Lymphocyte-specific protein 1	-0.123	0.022
H2-Q7	H-2 class I histocompatibility antigen, Q7 alpha chain	-0.166	0.008
Bcl7a	B-cell CLL/lymphoma 7 protein family member A	-0.216	0.004
Traf2	TNF receptor-associated factor 2	0.024	0.053
Irgm1	Immunity-related GTPase family M protein 1	-0.060	0.070
Manf	Mesencephalic astrocyte-derived neurotrophic factor	-0.149	0.073
Fcrla	Fc receptor-like A	-0.020	0.075
H2-K1	H-2 class I histocompatibility antigen, K-B alpha chain	-0.047	0.098
HVM51	Ig heavy chain V region AC38 205.12	0.077	0.098
IGKC	Ig kappa chain C region	0.220	0.100
Cd19	B-lymphocyte antigen CD19	0.057	0.109
Bank1	B-cell scaffold protein with ankyrin repeats	-0.023	0.111
Cd79b	B-cell antigen receptor complex-associated protein beta chain	0.077	0.125
Ncf4	Neutrophil cytosol factor 4	0.077	0.130
H2-Ab1	H-2 class II histocompatibility antigen, A beta chain	0.153	0.132
Cct4	T-complex protein 1 subunit delta	-0.020	0.151
Irf4	Interferon regulatory factor 4	0.037	0.152
Fcrl5	Fc receptor-like protein 5	0.044	0.153
Ms4a1	B-lymphocyte antigen CD20	0.053	0.171
H2-D1	H-2 class I histocompatibility antigen, D-B alpha chain	-0.044	0.181
Cd86	T-lymphocyte activation antigen CD86	-0.053	0.235
Nkrf	NF-kappa-B-repressing factor	-0.046	0.252
Cd72	B-cell differentiation antigen CD72	0.014	0.259
Cct6a	T-complex protein 1 subunit zeta	-0.027	0.266
Tlr9	Toll-like receptor 9	0.027	0.289
Hells	Lymphocyte-specific helicase	-0.030	0.299
H2-D1	H-2 class I histocompatibility antigen, D-D alpha chain	0.147	0.305
Fcer2	Low affinity immunoglobulin epsilon Fc receptor	0.040	0.313
Ly6e	Lymphocyte antigen 6E	0.237	0.338
Leng8	Leukocyte receptor cluster member 8 homolog	-0.412	0.375

Supplementary Table 3. Proteasomal proteins in LPS stimulated B cells

Protein symbol	Protein name	Fold change	P-value
Adrm1	Proteasomal ubiquitin receptor ADRM1	0.4433	0.0057
Psmb4	Proteasome subunit beta type-4	0.0432	0.0177
Cdc37	Hsp90 co-chaperone Cdc37	0.0430	0.0086
Otub1	Ubiquitin thioesterase OTUB1	0.0399	0.0233
Psmd8	26S proteasome non-ATPase regulatory subunit 8	-0.0667	0.0624
Psma4	Proteasome subunit alpha type-4	-0.0401	0.0640
Usp5	Ubiquitin carboxyl-terminal hydrolase 5	0.0965	0.0300
Ubfd1	Ubiquitin domain-containing protein UBFD1	-0.0564	0.0148
Rnf213	E3 ubiquitin-protein ligase RNF213	-0.0603	0.0057
Ube2v1	Ubiquitin-conjugating enzyme E2 variant 1	-0.0634	0.0306
Usp48	Ubiquitin carboxyl-terminal hydrolase 48	-0.1198	0.0369
Ube2v2	Ubiquitin-conjugating enzyme E2 variant 2	-0.1928	0.0120
Ube2l3	Ubiquitin-conjugating enzyme E2 L3	-0.2397	0.0160
Ube2n	Ubiquitin-conjugating enzyme E2 N	-0.0802	0.0820
Ube2d3	Ubiquitin-conjugating enzyme E2 D3	-0.0364	0.0853
Psmc1	26S protease regulatory subunit 4	0.0296	0.0861
Usp25	Ubiquitin carboxyl-terminal hydrolase 25	0.0636	0.0863
Ube2z	Ubiquitin-conjugating enzyme E2 Z	-0.0464	0.0888
Trim31	E3 ubiquitin-protein ligase TRIM31	-0.0431	0.0928
Psme1	Proteasome activator complex subunit 1	-0.0534	0.1064
Rnf40	E3 ubiquitin-protein ligase BRE1B	0.0469	0.1133
Rbx1	E3 ubiquitin-protein ligase RBX1	-0.0629	0.1247
Rnf2	E3 ubiquitin-protein ligase RING2	-0.0263	0.1365
Cops7a	COP9 signalosome complex subunit 7a	-0.0461	0.1387
Hectd1	E3 ubiquitin-protein ligase HECTD1	0.0400	0.1450
Psmb1	Proteasome subunit beta type-1	-0.0434	0.1454
Usp3	Ubiquitin carboxyl-terminal hydrolase 3	0.0604	0.1465
Uba1	Ubiquitin-like modifier-activating enzyme 1	0.0361	0.1523
Uchl5	Ubiquitin carboxyl-terminal hydrolase isozyme L5	-0.0566	0.1524
Psmb5	Proteasome subunit beta type-5	0.0203	0.1720
Psmd14	26S proteasome non-ATPase regulatory subunit14	-0.0201	0.1784
Psma7	Proteasome subunit alpha type-7	-0.0236	0.1805
Psma6	Proteasome subunit alpha type-6	-0.0236	0.2140
Psmd10	26S proteasome non-ATPase regulatory subunit10	-0.0329	0.2193
Psmd6	26S proteasome non-ATPase regulatory subunit 6	-0.0202	0.2246
Ube2q1	Ubiquitin-conjugating enzyme E2 Q1	0.0370	0.2482
Hsp90a	Heat shock protein HSP 90-alpha	-0.0139	0.2627
Psmb10	Proteasome subunit beta type-10	-0.0366	0.2647
Psmd3	26S proteasome non-ATPase regulatory subunit 3	0.0198	0.3020
Psmd11	26S proteasome non-ATPase regulatory subunit 11	0.0165	0.3500

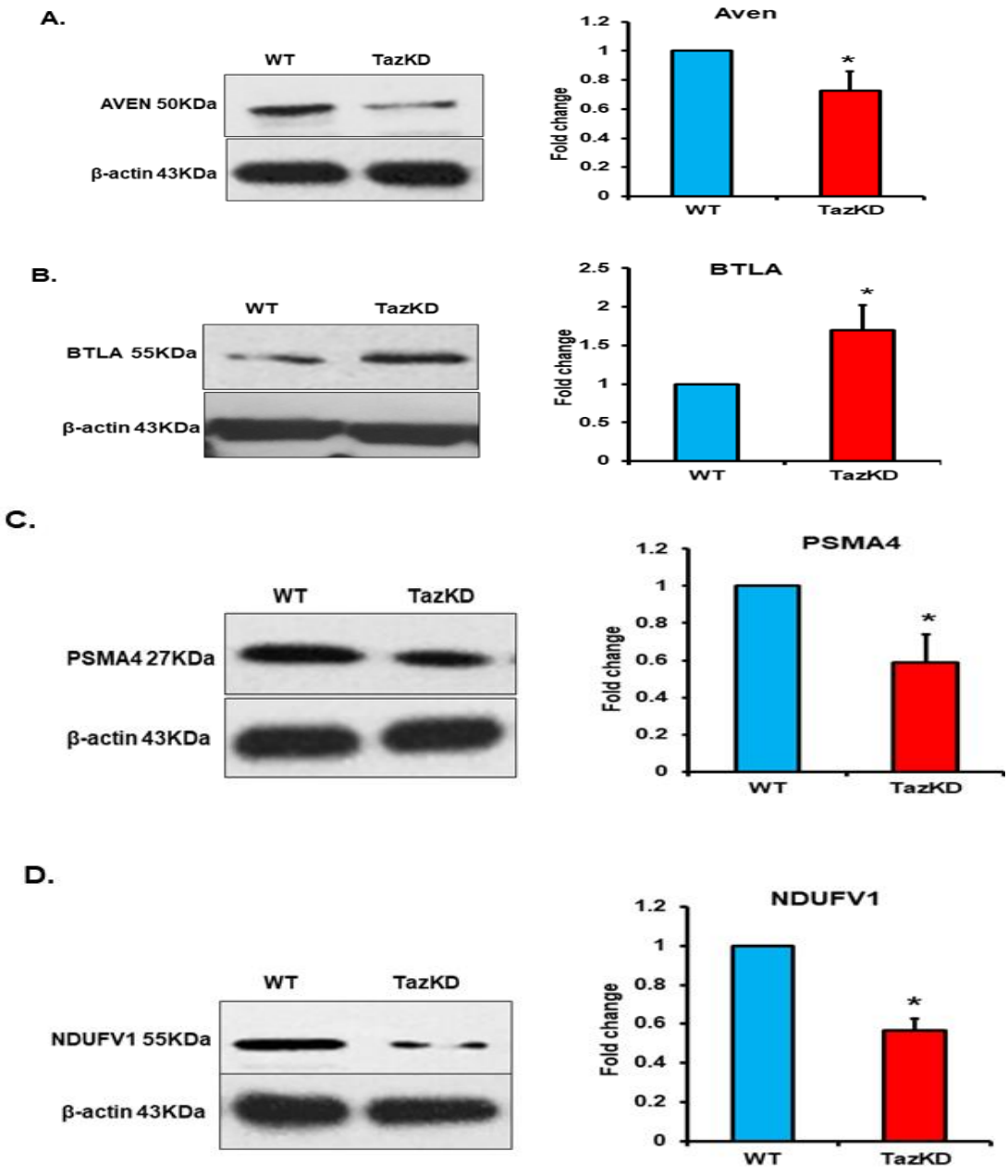
Supplementary Table 4. Mitochondrial proteins in LPS stimulated B cells

Protein symbol	Protein name	Fold change	P-value
Carkd	ATP-dependent (S)-NAD(P)H-hydrate dehydratase	0.1838	0.0097
Dars2	Aspartate--tRNA ligase, mitochondrial	0.1039	0.0312
Acadl	Long-chain specific acyl-CoA dehydrogenase, Mitochondrial	0.0865	0.0274
Echs1	Enoyl-CoA hydratase, mitochondrial	0.0801	0.0069
Acsf2	Acyl-CoA synthetase family member 2, mitochondrial	0.0665	0.0064
Pcca	Propionyl-CoA carboxylase alpha chain, mitochondrial	0.0601	0.0458
Isoc2a	Isochorismatase domain-containing protein 2A,	0.0535	0.0210
Hibch	3-hydroxyisobutyryl-CoA hydrolase, mitochondrial	0.0502	0.0278
Me2	NAD-dependent malic enzyme, mitochondrial	0.0500	0.0320
Aldh2	Aldehyde dehydrogenase, mitochondrial	0.0495	0.0147
Gpd2	Glycerol-3-phosphate dehydrogenase, mitochondrial	0.0466	0.0030
Msra	Mitochondrial peptide methionine sulfoxide reductase	0.0435	0.0035
Ddx51	ATP-dependent RNA helicase DDX51	0.0371	0.0237
Slc25a3	Phosphate carrier protein, mitochondrial	0.0363	0.0072
Atp5b	ATP synthase subunit beta, mitochondrial	0.0327	0.0156
Hadhb	Trifunctional enzyme subunit beta, mitochondrial	0.0298	0.0439
Slc25a12	Calcium-binding mitochondrial carrier protein Aralar1	0.0267	0.0241
Atp5o	ATP synthase subunit O, mitochondrial	0.0264	0.0294
Ddx1	ATP-dependent RNA helicase DDX1	0.0231	0.0464
Abcf2	ATP-binding cassette sub-family F member 2	-0.0236	0.0376
Mpc2	Mitochondrial pyruvate carrier 2	-0.0397	0.0024
Mrpl11	39S ribosomal protein L11, mitochondrial	-0.0398	0.0397
Mrps16	28S ribosomal protein S16, mitochondrial	-0.0461	0.0499
Gcdh	Glutaryl-CoA dehydrogenase, mitochondrial	-0.0528	0.0371
Pnpt1	Polyribonucleotide nucleotidyl transferase 1	-0.0532	0.0284
Ndufv1	NADH dehydrogenase [ubiquinone] flavoprotein 1	-0.0533	0.0132
Coa7	Cytochrome c oxidase assembly factor 7	-0.0593	0.0001
Pam16	Mitochondrial import inner membrane translocase subunit TIM16	-0.0659	0.0312
Mrpl53	39S ribosomal protein L53, mitochondrial	-0.0827	0.0057
Chchd2	Coiled-coil-helix-coiled-coil-helix domain-containing protein 2, mitochondrial	-0.0861	0.0387
Tomm7	Mitochondrial import receptor subunit TOM7	-0.1025	0.0398
Mrpl22	Homolog 39S ribosomal protein L22	-0.1099	0.0076
Ndufa2	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2	-0.1129	0.0077
Atp5h	ATP synthase subunit d, mitochondrial	-0.1133	0.0117
Ppif			

Cycs	Peptidyl-prolyl cis-trans isomerase F, mitochondrial	-0.1228	0.0297
Atp5g1	Cytochrome c, somatic	-0.1299	0.0212
Hspe1	ATP synthase F(0) complex subunit C1, mitochondrial	-0.1702	0.0337
Cluh	10 kDa heat shock protein, mitochondrial	-0.1936	0.0495
	Clustered mitochondria protein homolog	-0.0401	0.0500
Tmem11	Transmembrane protein 11, mitochondrial	0.1104	0.0510
Atp5e	ATP synthase subunit epsilon, mitochondrial	-0.0400	0.0512
Hadha	Trifunctional enzyme subunit alpha, mitochondrial	0.0297	0.0514
Rexo2	Oligoribonuclease, mitochondrial	0.0636	0.0517
Rars2	Probable arginine--tRNA ligase, mitochondrial	0.0237	0.0521
Mrpl4	39S ribosomal protein L4, mitochondrial	0.0506	0.0556
Cpox	Oxygen-dependent coproporphyrinogen-III oxidase, Mitochondrial	0.1670	0.0620
Mrpl55	39S ribosomal protein L55, mitochondrial	0.2001	0.0631
Pitrm1	Presequence protease, mitochondrial	-0.0669	0.0642
Cmpk2	UMP-CMP kinase 2, mitochondrial	0.0302	0.0778
Ivd	Isovaleryl-CoA dehydrogenase, mitochondrial	0.0300	0.0852
Mrrf	Ribosome-recycling factor, mitochondrial	0.0272	0.0906
Uqcrb	Cytochrome b-c1 complex subunit 7	-0.0694	0.0917
Glud1	Glutamate dehydrogenase 1, mitochondrial	0.0398	0.0919
Prkaa1	5'-AMP-activated protein kinase catalytic subunit alpha-1	0.0301	0.0977
Mrps26	28S ribosomal protein S26, mitochondrial	0.0536	0.1012
Mthfd2	Bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase, mitochondrial	-0.0664	0.1031
Ndufs3	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	0.0700	0.1040
Tufm	Elongation factor Tu, mitochondrial	-0.0104	0.1107
Uqcr10	Cytochrome b-c1 complex subunit 9	0.0602	0.1110
Oat	Ornithine aminotransferase, mitochondrial	-0.0634	0.1137
Nnt	NAD(P) transhydrogenase, mitochondrial	0.0536	0.1145
Mrpl13	39S ribosomal protein L13, mitochondrial	-0.0364	0.1146
Dld	Dihydrolipoyl dehydrogenase, mitochondrial	0.0301	0.1147
Bcs1l	Mitochondrial chaperone BCS1	-0.0727	0.1169
Cox6b1	Cytochrome c oxidase subunit 6B1	0.0735	0.1236
Atpif1	ATPase inhibitor, mitochondrial	-0.1559	0.1253
Mrpl40	39S ribosomal protein L40, mitochondrial	-0.0327	0.1283
Acadvl	Very long-chain specific acyl-CoA dehydrogenase,	0.0533	0.1286
Mrpl46	39S ribosomal protein L46, mitochondrial	0.1269	0.1294
Mrpl30	39S ribosomal protein L30, mitochondrial	0.0237	0.1294
Gpx4	Phospholipid hydroperoxide glutathione peroxidase, mitochondrial	-0.0198	0.1297

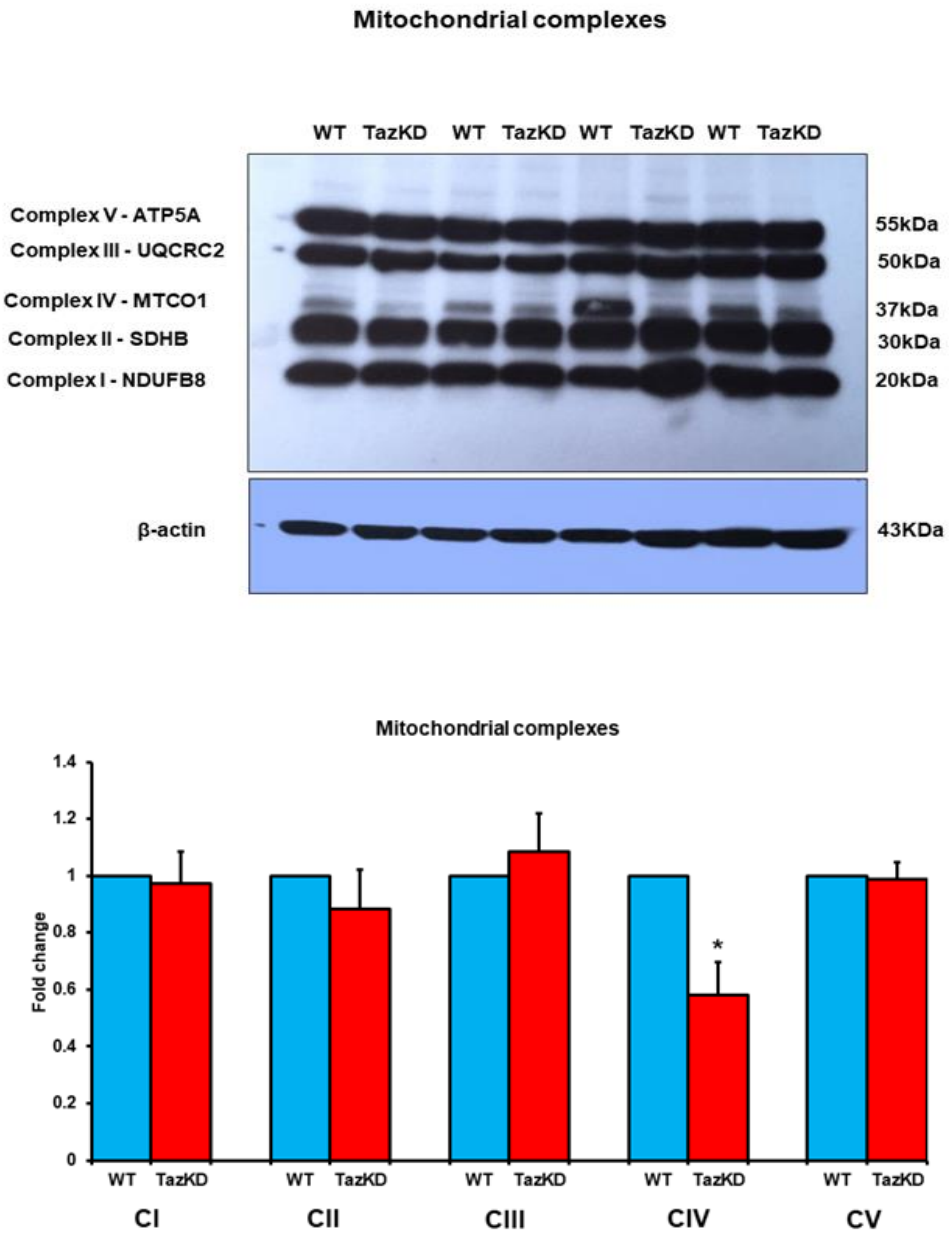
Mut	Methylmalonyl-CoA mutase, mitochondrial	0.0403	0.1313
Pmpcb	Mitochondrial-processing peptidase subunit beta	0.0370	0.1316
Atp5i	ATP synthase subunit e, mitochondrial	-0.0434	0.1330
Prdx3	Thioredoxin-dependent peroxide reductase, mitochondrial	-0.0599	0.1350
Ndufs6	NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial	-0.0260	0.1361
Acat1	Acetyl-CoA acetyltransferase, mitochondrial	0.0199	0.1404
Mtor	Serine/threonine-protein kinase mTOR	0.1242	0.1407
Pdhx	Pyruvate dehydrogenase protein X component, mitochondrial	-0.0661	0.1435
Tomm20	Mitochondrial import receptor subunit TOM20 homolog	-0.0364	0.1452
Pmpca	Mitochondrial-processing peptidase subunit alpha	-0.0364	0.1453
Suclg1	Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial	-0.0401	0.1454
Aldh4a1	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	0.0170	0.1454
Mp68	6.8 kDa mitochondrial proteolipid	-0.0663	0.1462
Coa3	Cytochrome c oxidase assembly factor 3 homolog, mitochondrial	-0.1360	0.1494
Ddx10	Probable ATP-dependent RNA helicase DDX10	-0.0266	0.1517
Ndufa7	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	-0.0296	0.1555
Slc25a1	Tricarboxylate transport protein, mitochondrial	-0.0631	0.1557
Ndufa10	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial	0.0333	0.1604
Ndufa6	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6	-0.0332	0.1621
Hadh	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	0.0701	0.1625
Ndufb10	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	0.0268	0.1626
Ptrh2	Peptidyl-tRNA hydrolase 2, mitochondrial	0.0635	0.1662
Aco2	Aconitate hydratase, mitochondrial	0.0196	0.1667
Mrps31	28S ribosomal protein S31, mitochondrial	-0.0199	0.1669
Mavs	Mitochondrial antiviral-signaling protein	-0.0595	0.1689
Lactb	Serine beta-lactamase-like protein LACTB, mitochondrial	0.0501	0.1707
Mrpl15	39S ribosomal protein L15, mitochondrial	-0.0562	0.1713
Fis1	Mitochondrial fission 1 protein	-0.0565	0.1784

Supplementary Figure 1



Supplementary Figure 1. Validation of some proteins in LPS stimulated B cells. A. Aven (survival marker). **B.** BTLA (immunogenic marker). **C.** PSMA4 (proteasomal marker). **D.** NDUFV1 (mitochondrial marker). n=3, *p<0.05 compared with WT B cells.

Supplementary Figure 2



Supplementary Figure 2. Western blot of mitochondrial oxidative phosphorylation complexes. Mitochondrial complex protein levels were measured in whole cell lysates of LPS stimulated cells. An antibody cocktail was used against respiratory complex subunits as following: NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8 (NDUFB8) of complex I, succinate dehydrogenase subunit B (SDHB) of Complex II, cytochrome c oxidase subunit 1 (MTCO1) of Complex IV, cytochrome b-c1 complex subunit 2 (UQCRC2) of Complex III and

ATP synthase subunit alpha (ATP5A) of Complex V. The protein levels were normalized to β-Actin. n=3, *p<0.05 compared with WT B cells.

CHAPTER V. Tafazzin deficiency attenuates anti-cluster of differentiation 40 and interleukin-4 activation of mouse B lymphocytes

Rationale: In chapter III, we reported that Taz deficiency in B cells resulted in impairment in mitochondrial function and glycolysis. In addition, we found that B cells function and proliferation were inhibited in TazKD B cells after activation with LPS. Impairment of metabolic pathways in B cells may result in a decrease in their activity after stimulation. Moreover, we reported that the 26S proteasome and immunoproteasome activities were reduced in naïve and LPS stimulated TazKD B cells. Mitochondrial function is required for the proteasome system and decreased ATP production inhibits proteasome activity. That study was the first study to report the impact of Taz deficiency in B cells activity via a T cell-independent antigen activation. B cells can be activated through either T cell-independent antigens or T cell-dependent antigens. In the T cell-dependent pathway, the B cells require T help through CD40L interaction and cytokines such as IL-4 to become activated. Therefore, we investigated the effect of Taz deficiency on B cells stimulated with T cell-dependent antigens. In this chapter, we isolated B cells from WT and TazKD mice and stimulated them with CD40+IL-4 and examined their immunological properties.

Hypothesis: Taz deficiency inhibits metabolic activities and function in CD40+IL-4 stimulated TazKD B cells.

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Tafazzin deficiency attenuates anti-cluster of differentiation 40 and interleukin-4 activation of mouse B lymphocytes

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Key words: B lymphocytes; tafazzin; T cell signaling; Barth Syndrome; cardiolipin; mitochondria

5.1 Abstract

Barth Syndrome (BTHS) is a rare X-linked genetic disease caused by mutations in TAFAZZIN. The tafazzin (Taz) protein is a cardiolipin remodeling enzyme required for maintaining mitochondrial function. Patients with BTHS exhibit impaired mitochondrial respiratory chain and metabolic function and are susceptible to serious infections. B lymphocytes (B cells) play a vital role in humoral immunity required to eradicate circulating antigens from pathogens. Intact mitochondrial respiration is required for proper B cell function. We investigated whether Taz deficiency in mouse B cells altered their response to activation by anti-cluster of differentiation 40 (anti-CD40) + interleukin-4 (IL-4). B cells were isolated from 3-4-month-old wild type (WT) or tafazzin knockdown (TazKD) mice and stimulated with anti-CD40 + IL-4 for 24 h and cellular bioenergetics, surface marker expression, proliferation, antibody production, and proteasome and immunoproteasome activities determined. TazKD B cells exhibited reduced mRNA expression of Taz, lowered levels of cardiolipin and impairment in both oxidative phosphorylation and glycolysis compared to WT B cells. In addition, anti-CD40 + IL-4 stimulated TazKD B cells expressed lower levels of the immunogenic surface markers, cluster of differentiation 86 (CD86) and cluster of differentiation 69 (CD69), exhibited a lower proliferation rate, reduced production of immunoglobulin M and immunoglobulin G, and reduced proteasome and immunoproteasome proteolytic activities compared to WT B cells stimulated with anti-CD40 + IL-4. The results indicate that Taz is required to support T-cell dependent signaling activation of mouse B cells.

5.2 Introduction

Barth Syndrome (BTHS) is a rare (1:300,000) X-linked genetic disorder that affects young males (Hauff and Hatch 2006; Clarke et al. 2013). BTHS is characterized by cardiomyopathy, neutropenia, skeletal myopathy and neutropenia. Heart failure and recurrent bacterial infections are the main causes of death in BTHS patients (Raja et al. 2017). BTHS is

caused by mutations in the TAFAZZIN gene which results in a dysfunctional or absent tafazzin (Taz) protein (Adès et al. 1993; Bione et al. 1996). The Taz protein exhibits a transacylase enzymatic activity essential for the terminal maturation of the mitochondrial phospholipid cardiolipin (CL) (Xu et al. 2006). CL is a major mitochondrial phospholipid found exclusively in mitochondrial membranes. CL modulates the activity of many key enzymes of the mitochondrial respiratory chain and plays a critical role in mitochondrial bioenergetics by acting as a 'glue' that holds together and stabilizes electron transport chain complexes (Zegallai and Hatch 2021; Paradies et al. 2014). The appropriate content and molecular acyl species composition of CL are essential for proper mitochondrial activity and maintenance of cellular function (Hauff and Hatch 2006; Shi 2010).

As a multisystem metabolic disorder many BTHS patients exhibit susceptibility to serious infections. The most common reported cause of immunodeficiency in BTHS patients is neutropenia (Clarke et al. 2013). However, the status of other immune cells in BTHS patients has not been thoroughly investigated. B lymphocytes play a crucial role in the immune system by regulating the immune response in both normal and pathological situations (LeBien and Tedder 2008). In addition, B lymphocytes mediate humoral immunity in the adaptive immune system and are responsible for antibody production (Cantrell 2015). B lymphocytes modulate function of other immune cells including neutrophils through synthesis and secretion of cytokines and chemokines. For example, B lymphocytes produce CXCL1 (growth-regulated oncogene-alpha) which is chemotactic for neutrophils (Hu et al. 2004; Dai et al. 2012). Thus, B lymphocyte dysfunction may contribute to immune dysfunction.

Naive B cells are maintained in a quiescent state with low metabolic activity until activated by antigen recognition. Activation of B cells through T cell dependent pathways result in a transition of B cells from a quiescent to an activation and proliferation state (Rush and Hodgkin 2001). In addition, activation of B cells induces B cells to undergo metabolic

reprogramming through increased glycolysis and oxidative phosphorylation; processes required to initiate the B cell immune response (Caro-Maldonado et al. 2014; Waters et al. 2018). Finally, T cell activation signaling during T cell-B cell interactions is required to regulate B cell differentiation (Rush and Hodgkin 2001). Thus, activation of B cells mediated by T cell-dependent antigens is essential to initiate the adaptive immune response. We recently reported that Taz deficiency in lipopolysaccharide (LPS) stimulated B cells, a T-cell independent form of activation, resulted in reduced metabolic activity, proliferation, immunological function, and proteasomal activity (Zegallai et al. 2021). In this study, we investigated the impact of Taz deficiency on the metabolic activity and function of B cells activated through a T cell-dependent activation.

5.3 Material and Methods

Experimental animals

All animals were housed in pathogen-free facility (12 h light/dark cycle) at the University of Manitoba and had free access to food and water. All experimental procedures performed in this study were approved by the University of Manitoba Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines and regulations. This study is reported in accordance with ARRIVE guidelines. Tafazzin knockdown (TazKD) mice were generated by mating transgenic male mice containing a doxycycline (dox) inducible tafazzin specific short-hair-pin RNA (B6.Cg-Gt(ROSA)26Sortm1(H1/tetO-RNAi:Taz,CAG tetR)Bsf/ZkhuJ with female C57BL/6J mice from Jackson Laboratory (Boston,MA). Tafazzin knock down was maintained postnatally by administering dox (625 mg of dox/kg of chow) as part of the rodent chow (Catalog no. TD.01306) from Harlan (Indianapolis, USA) as described previously (Acehan et al. 2011). Male offspring were weaned at 3 weeks of age onto the above diet and wild type (WT) animals additionally received the dox containing diet. Male mice were

used for the experiments at 3-4 months of age. Male mice positive for the tafazzin shRNA transgene were identified by PCR as described previously (Acehan et al. 2011).

Isolation and culture of B lymphocytes

Splenic naïve B lymphocytes were isolated by magnetic bead negative selection using EasySep^{TM} Mouse B Cell Isolation Kit (Catalog no. 19854) from STEMCELL Technologies (Vancouver, BC). B cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% antibiotic/antimycotic, and 2-mercaptoethanol at 37°C with 5% CO_2. Subsequent to isolation B cells were stimulated or not with 1 μg/ml anti-CD40 (catalog no. 553721; BD Biosciences) together with 5 ng/ml recombinant murine IL-4 (catalog no. 214-14; PeproTech). B cells were used immediately for experiments after isolation from WT and TazKD animals and dox was not included in the culture medium. Unless otherwise indicated, all other reagents used were of analytical grade and were obtained from either Thermo Fisher Scientific (Winnipeg, MB) or Sigma-Aldrich (Oakville, ON).

Assessment of cellular bioenergetics

Cellular bioenergetics was assessed by measuring oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in activated B cells using a Seahorse XF24 Extracellular Flux Analyzer (Agilent Technologies) with kits from Agilent (Santa Clara, CA). Briefly, isolated WT and TazKD B cells were stimulated with anti-CD40 + IL-4 for 24 h. Anti-IgM $F(ab')_2$ was added at concentration of 10 μg/ml to increase the glycolysis rate (catalog no. 115-006-020; Jackson ImmunoResearch). It was reported that activation of B cells with anti-IgM amplifies the glycolysis signal and increases the assay sensitivity (Waters et al. 2018). The cells washed with either OCR or ECAR assay medium then seeded in XF24 24-well plates coated with Cell-Tak from BD Biosciences (Mississauga, ON) at a density of 4×10^5 cells per well. The Cell-Tak (1.12 μg/well) was used to generate an adherent monolayer of B cells on

the XF24 24-well plate. For mitochondrial function stress test (OCR assay) the following final concentrations of drugs were used: 1 μM oligomycin, 1 μM FCCP, 1 μM antimycin A, and 1 μM rotenone as per the manufacturer's instructions. For glycolytic function test (ECAR assay) the following final concentrations of drugs were used: 10 μM glucose, 1 μM oligomycin, and 25 μM 2-deoxyglucose as per the manufacturer's instructions. The OCR and ECAR values were normalized to the cell number in each well.

B lymphocytes surface marker analysis

Cell surface markers expression of differentiation 86 (CD86) and cluster of differentiation 69 (CD69) were measured using flow cytometry were measured in WT and TazKD B cells using flow cytometry. 2×10^6 B cells were stimulated with anti-CD40 + IL-4 for 24 h and cultured in 1 ml/well in 24-well plate at 37°C in 5% humidified CO_2. For staining, 2×10^6 cells were incubated with APC-conjugated anti-cluster of differentiation 19 (CD19) in combination with either PE-A labelled anti-CD69 or PE-CY7-A labelled anti-CD86 antibodies for 10-15 minutes at 4°C. After washing the cells, the cells were analyzed by flow cytometry using a BD FACSCanto II instrument. FlowJo software was used for data analysis. Anti-CD19, anti-CD69, anti-CD86 were obtained from BD Biosciences (Mississauga, ON).

Analysis of cell proliferation

Proliferating and apoptotic cells were determined by cell cycle analysis using the Nicoletti assay (Riccardi and Nicoletti 2006). B cells were stimulated with anti-CD40 + IL-4 for 24 h and cultured in 24-well plates. The cells were washed two times with PBS and suspended in 100 μl of ice cold PBS. Cells were then fixed in 5 ml of 70% ice-cold ethanol. After fixation the cells were stained with DNA fluorescent dye that quantitatively stains the DNA (40 μg/ml propidium iodide, 0.5 mg/ml RNase A, 0.1% Triton X-100, 1% sodium citrate) for 1 h at 4°C in the dark. Then flow cytometry analysis was performed to detect red fluorescence (460 nm)

for 10,000 cells using an Attune NxTModel AFC2 flow cytometer (Thermo Fisher Scientific). The number of proliferating cells was determined by counting the number of cells entering the S/G2 phase from the G0/G1 phase (non-proliferating phase). The percentage of apoptotic nuclei in sub-G1 section, healthy nuclei in G0/G1 section, and proliferating nuclei in S/G2 section were estimated by analysis of the DNA histogram and statistically compared between the samples (Riccardi and Nicoletti 2006).

Measurement of antibodies

WT and TazKD B cells were stimulated with anti-CD40 + IL-4 for 72 h. After incubation the supernatant fractions were collected, and immunoglobulin G (IgG) and immunoglobulin M (IgM) concentration determined using isotype-specific enzyme-linked immunosorbent assay (ELISA) kits as per the manufacturer's instructions. The IgM mouse uncoated ELISA kit (Catalog no.88-50470-22) and IgG (total) mouse uncoated ELISA kit (Catalog no.88-50400-22) were obtained from ThermoFisher Scientific (Winnipeg, MB).

Proteasome and immunoproteasome activity assays.

Proteasome activity was assessed by measuring the 20S subunit activity using fluorescent substrate-SUC-LLVY-AMC (Catalog no. 10008041) from Cayman Chemicals (Ann Arbor, MI). The fluorescent intensity was read at excitation=360 nm and emission=480nm. Immunoproteasome activity was determined by measuring the proteolytic activity of β1i/LMP2with S310 (Ac-PAL-AMC) as a fluorescent substrate using a commercial kit (Catalog no. 10008041) from Cayman Chemicals (Ann Arbor, MI). The fluorescent intensity was read at excitation = 345nm and emission=445nm.

Relative Gene Expression.

RNA was isolated from WT B cells and TazKD B cells using RNeasy Mini Kit and Qiashredder homogenizer columns. RT-PCR was performed using the QuantiTect® Probe RT PCR Kit and

the double stranded DNA stain SYBR green as indicated by the manufacturer. Relative gene expression analysis was calculated using the 2-ΔΔCt method. The primers that used for RT-PCR detection as follow: tafazzin: forward, 5′-ACTCAGAACCAACCCACAGG-3′; reverse, 5′-CTGCTGAGTGGTCAGTGCAT-3′; β-actin: forward, 5′-GGCCAGGTCACTATTG -3′; reverse, 5′-GAGGTCTTTACGGATGTCAAC-3′. All primers were ordered from Integrated DNA Technologies.

Lipid Mass Spectrometry

Electrospray ionization - mass spectrometry coupled to high performance liquid chromatography was performed to quantify CL mass and fatty acyl molecular species as previously described (Sparagna et al. 2005).

Statistical analysis

Data were expressed as mean ± SD. Two tailed unpaired Student's t test was used to compare between two groups, one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test was performed to compare between multiple groups. A p value of < 0.05 was considered statistically significant.

5.4 Results

Taz-deficiency impairs cellular energy metabolism in anti-CD40 + IL-4 activated B cells

The relative expression of Taz mRNA was reduced (85% decrease, $p<0.05$) in TazKD B cells compared to WT B cells and this level of reduction in Taz mRNA was retained in anti-CD40 + IL-4 activated TazKD B cells compared to anti-CD40 + IL-4 activated WT B cells (Supplementary Fig. 1A). In addition, the reduced expression of Taz mRNA in TazKD B cells was correlated with a reduction (55% decrease, $p<0.05$) in CL and most molecular species of CL (Supplementary Fig. 1B &1C).

Intracellular energy metabolism is required for B cell differentiation and function. Activation of B cells augments cellular metabolic activity including mitochondrial oxidative

phosphorylation and glycolysis (Caro-Maldonado et al. 2014; Waters et al. 2018). Anti-CD40 + IL-4 activated TazKD B cells exhibited reduced basal respiration (16% decrease, $p<0.0001$), maximal respiration (10% decrease, $p<0.01$), and spare respiratory capacity (23% decrease, $p<0.05$) compared to anti-CD40 + IL-4 activated WT B cells (Figure 1A). In addition, anti-CD40 + IL-4 activated TazKD B cells exhibited reduced glycolysis (28% decrease, $p<0.0001$), glycolytic capacity (42% decrease, $p<0.0001$), and glycolytic reserve (25% decrease, $p<0.0001$) compared to anti-CD40 + IL-4 activated WT B cells (Figure 1B). Thus, Taz deficiency in anti-CD40 + IL-4 activated B cells impairs both mitochondrial respiration and glycolytic function.

Figure 1

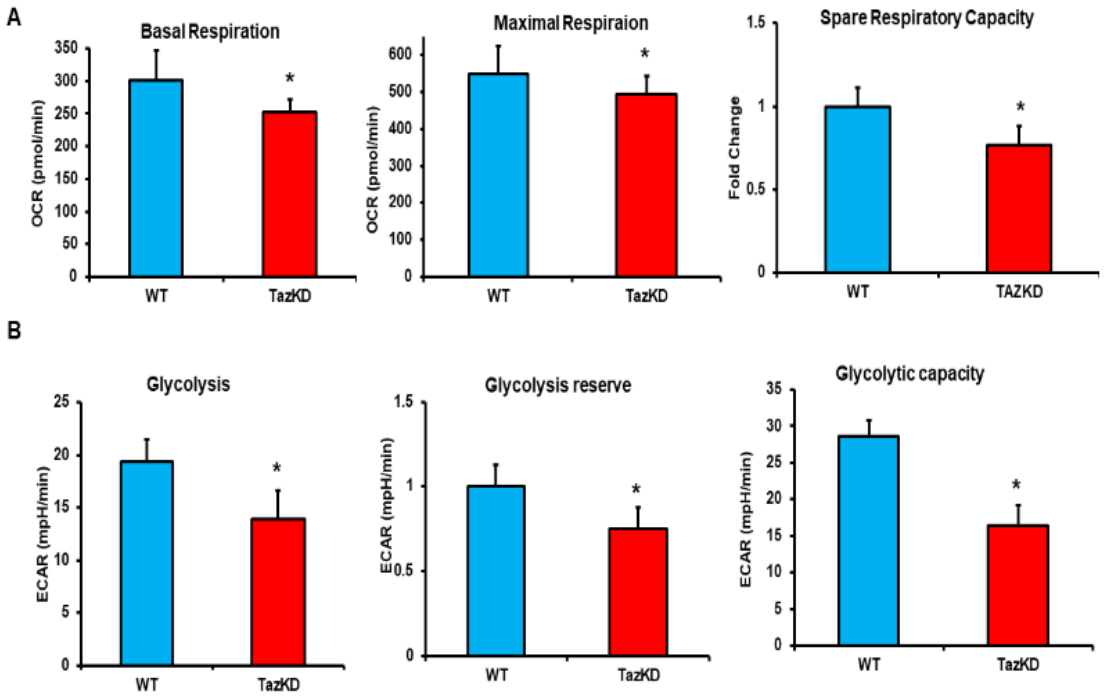

Figure 1. Taz-deficiency results in impairment of cellular metabolism in anti-CD40 + IL-4 activated B cells. WT or TazKD B cells were stimulated with anti-CD40 + IL-4 for 24 h and mitochondrial function and glycolytic activity determined as described in Materials and Methods. **A.** Basal respiration, maximal respiration and spare respiratory capacity. **B.** Glycolysis, glycolytic capacity and glycolytic reserve. N=3, *p<0.05 compared with WT B cells.

Taz-deficiency reduces proliferation of anti-CD40 + IL-4 activated B cells

The ability of B cells to proliferate after T-cell contact signaling is essential for a rapid immune response. Murine B cells proliferate when exposed to anti-CD40 + IL-4 *in vitro* (Griebel et al. 1999) . We analyzed the impact of Taz deficiency on naïve and anti-CD40 + IL-4 activated B cell proliferation by measuring the percentage of cells in each stage of cell cycle including Sub-G1, G0/G1 and S/G2 phases. There were no significant differences between naïve TazKD B cells and naïve WT B cells at any stage of the cell cycle (Figure 2A). In contrast, anti-CD40 + IL-4 activated TazKD B cells exhibited an increase in the percentage of cells in the Sub-G1 phase (6% increase, $p<0.05$) and in the G0/G1 phase (13%, $p<0.05$) compared to anti-CD40 + IL-4 activated WT B cells (Figure 2B). In addition, the percentage of B cells in the S/G2 phase was decreased in anti-CD40 + IL-4 activated TazKD B cells (10% decrease, $p<0.05$) compared to anti-CD40 + IL-4 activated WT B cells (Figure 2B). Thus, Taz-deficiency reduces proliferation of anti-CD40 + IL-4 activated B cells.

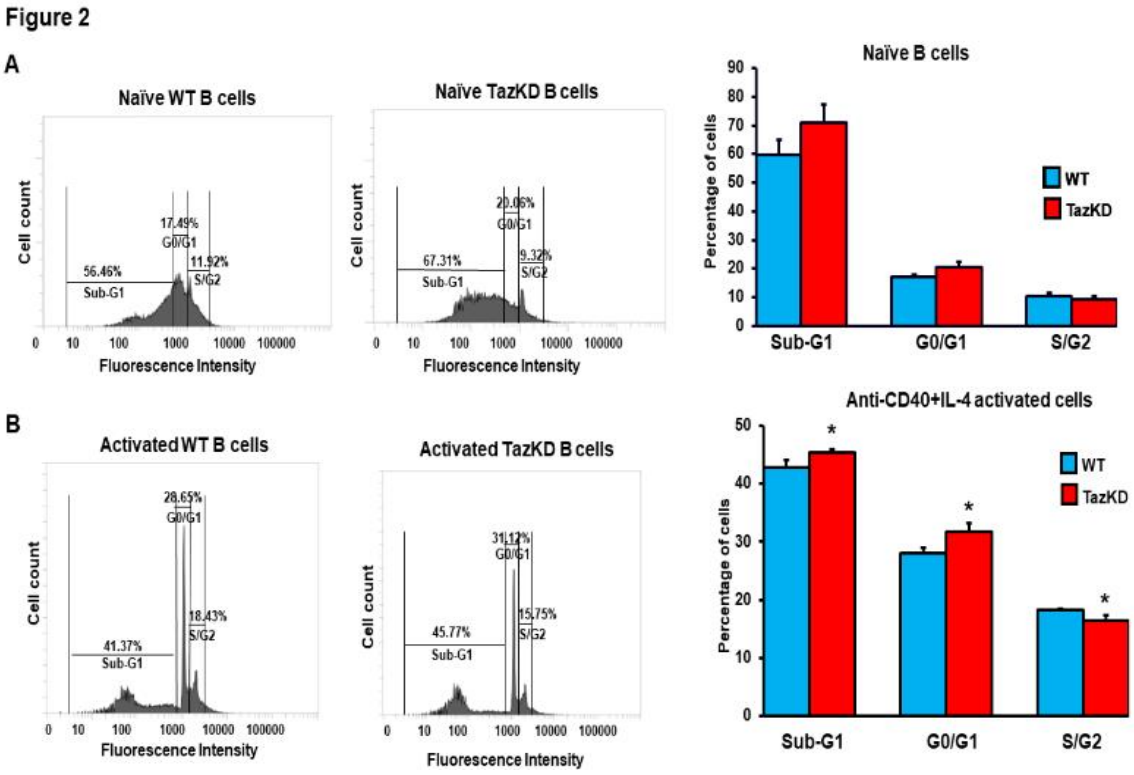

Figure 2. Taz-deficiency results in reduced proliferation in anti-CD40 + IL-4 activated B cells. Splenic B cells were stimulated for 24 h with anti-CD40 + IL-4 and the percentage of cells in Sub-G1, G0/G1 and S/G2 phases of the cell cycle were measured using flow cytometry. **A.** Flow cytometry images show the percentage of cells in Sub-G1, G0/G1 and S/G2 in naïve WT and TazKD B cells. **B.** Flow cytometry images show the percentage of cells in Sub-G1, G0/G1 and S/G2 in anti-CD40 + IL-4 activated WT and TazKD B cells. N=3-5, *p<0.05 compared with WT B cells.

Taz-deficiency reduces IgM and IgG production in anti-CD40 + IL-4 activated B cells

Activated B cells synthesize and secrete IgM and IgG antibodies. We examined the impact of Taz deficiency on the secretion of IgM and IgG in anti-CD40 + IL-4 activated B cells. Anti-CD40 + IL-4 activated TazKD B cells exhibited a decrease in IgM (15% decrease, $p<0.05$) and IgG (35% decrease, $p<0.05$) secretion into the medium compared to anti-CD40 + IL-4 activated WT B cells (Figure 3A, B). Thus, Taz-deficiency reduces IgM and IgG secretion from anti-CD40 + IL-4 activated B cells.

Figure 3

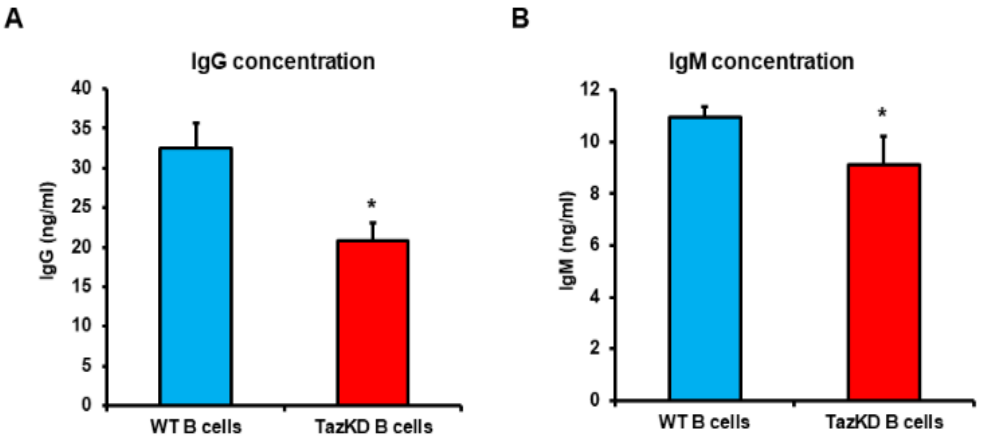

Figure 3. Taz-deficiency reduces IgM and IgG production in anti-CD40 + IL-4 activated B cells. WT and TazKD B cells were activated with CD40+IL-4 then the IgG and IgM levels were measured as described in Materials and Methods. **A.** IgG, **B.** IgM. n=4. *$p<0.05$ compared with WT B cells.

Taz-deficiency reduces proteasome and immunoproteasome activity in anti-CD40 + IL-4 activated B cells

The ubiquitin proteasome system plays a critical role in cellular processes including proliferation and differentiation. For example, optimal proteasomal activity is essential for immune cell function and homeostasis (Chen and Dou 2010). Previous studies have reported that mitochondrial dysfunction and metabolic activity impairment lead to inhibition of proteosome activity and assembly (Meul et al. 2020). We observed that both naïve and anti-CD40 + IL-4 activated TazKD B cells exhibited reduced proteasomal activity (50% decrease, $p<0.001$) compared to naïve and anti-CD40 +IL-4 activated WT B cells (Figure 4A, B). In addition, measured the activity of the immunoproteasome which is a specialized form of the proteasome that is constitutively expressed in immune cells (Eskandari et al. 2017). The immunoproteasome plays a key role in the immune response by breaking down antigen peptides and presenting them on the major histocompatibility complex I (Eskandari et al. 2017). We found that immunoproteasome activity was unaltered between naïve TazKD B cells and naïve WT B cells (Figure 4C). In contrast, immunoproteasome activity was reduced (50% decrease, $p<0.0001$) in anti-CD40 + IL-4 activated TazKD B cells compared to anti-CD40 + IL-4 activated WT B cells (Figure 4D). Thus, Taz-deficiency reduces proteasome and immunoproteasome activities in anti-CD40 + IL-4 activated B cells.

Figure 4

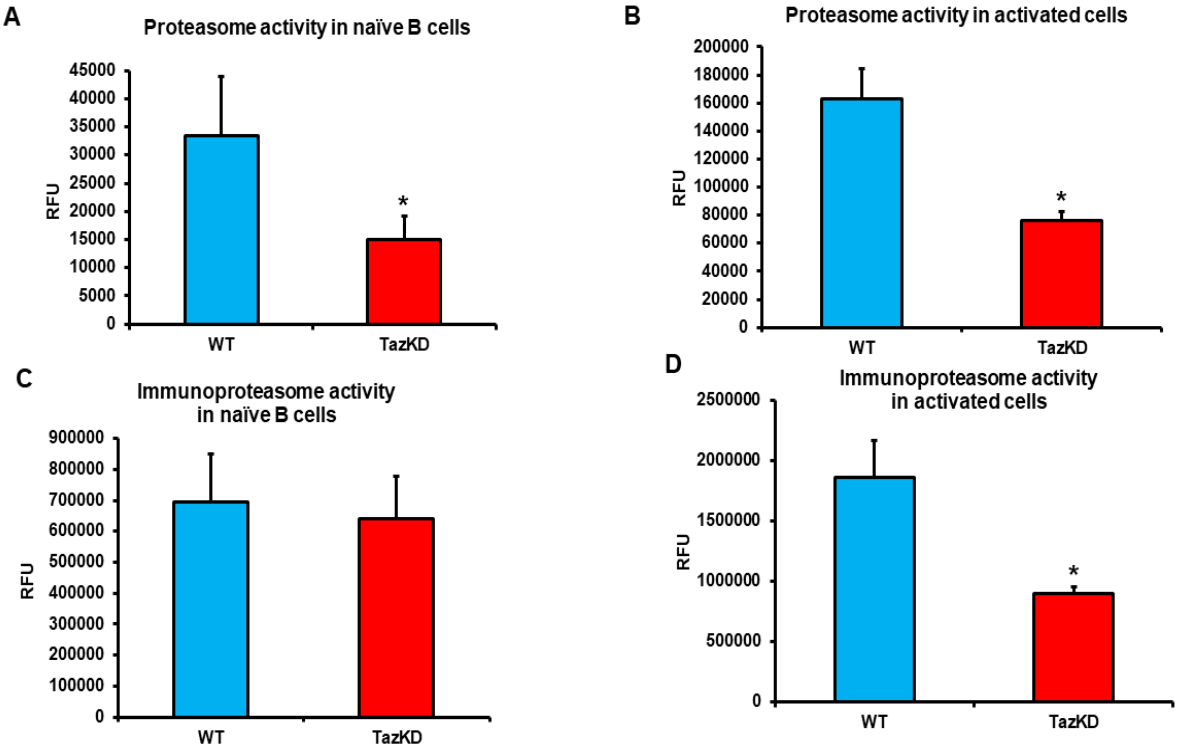

Figure 4. Taz-deficiency results in decreased proteasome and immunoproteasome activities in anti-CD40 + IL-4 activated B cells. Proteasome and immunoproteasome activities were measured in naïve and anti-CD40 + IL-4 activated WT and TazKD B cells as described in Materials and Methods. **A.** Proteasome activity in naïve WT and TazKD B cells. **B.** Proteasome activity in anti-CD40 + IL-4 activated WT and TazKD B cells. **C.** Immunoproteasome activity in naïve WT and TazKD B cells. **D.** Immunoproteasome activity inanti-CD40 + IL-4 activated WT and TazKD B cells. N=4-6, *p<0.05 compared with WT B cells.

Taz-deficiency reduces expression of CD86 and CD69 in anti-CD40 + IL-4 activated B cells

In the early stages of activation, B cells rapidly increase the expression of surface markers such as CD86 and CD69 which are required for interaction with other immune cells. For example, upregulation of CD86 on the B cell surface during activation is required for T cell-dependent B cell activation (Vazquez et al. 2009; Rau et al. 2009). We observed no significant difference in CD86 and CD69 expression between naïve WT and TazKD B cells (Figure 5A, B). In contrast, anti-CD40 + IL-4 activated Taz deficient B cells exhibited a decrease in both CD86 (40% decrease, $p<0.05$) and CD69 (15% decrease, $p<0.05$) compared to anti-CD40 + IL-4 stimulated WT B cells (Figure 5C, D). Thus, Taz-deficiency reduces expression of CD86 and CD69 in anti-CD40 + IL-4 activated B cells.

Figure 5

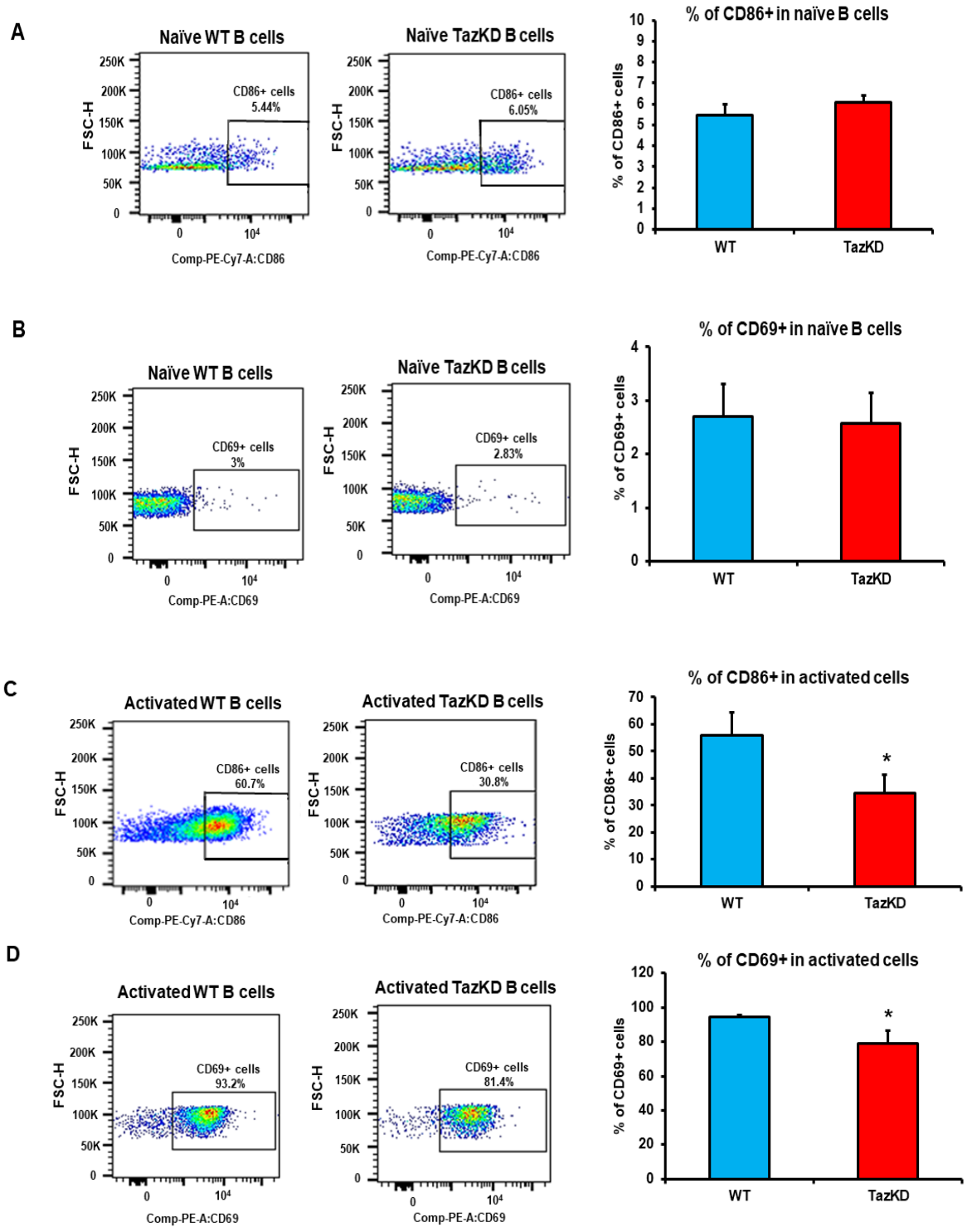

Figure 5. Taz-deficiency results in reduced CD86 and CD69 expression in anti-CD40 + IL-4 activated B cells. The expression of activation surface markers CD86 and CD69 were determined in naïve and anti-CD40 + IL-4 activated WT and TazKD B cells as described in

Materials and Methods. **A.** Flow cytometry images show the percentage of $CD86^+$ cells in naïve WT and TazKD B cells. **B.** Flow cytometry images show the percentage of $CD69^+$ cells in naïve WT and TazKD B cells. **C.** Flow cytometry images show the percentage of $CD86^+$ cells in anti-CD40 + IL-4 activated WT and TazKD B cells. **D.** Flow cytometry images show the percentage of $CD69^+$ cells in anti-CD40 + IL-4 activated WT and TazKD B cells. N=3, $*p<0.05$ compared with WT B cells.

5.5 Discussion

Barth Syndrome (BTHS) syndrome is a rare X-linked inherited multisystem disorder characterized by disturbance in mitochondrial function and energy production. Mitochondrial dysfunction in BTHS is due to mutation in the TAFAZZIN gene which encodes the CL-remodeling protein Taz. Taz deficiency may negatively impact immune cell function resulting in immunodeficiency and an increased susceptibility to life-threatening infections. Clinically, BTHS patients exhibit a neutropenia which provide a plausible explanation for why these patients exhibit increased susceptibility to infections (Steward et al. 2019). However, the potential contribution of other cells of the immune system to infections in BTHS is unknown.

Activated B cells play a key role in humoral immunity through the secretion of many antibodies and cytokines which alter and modulate the function of other immune cells including T cells and neutrophils (Cantrell 2015; Karmakar and Vermeren 2021). Two modes of B cell activation exist; T cell-independent and T cell-dependent (Cyster and Allen 2019). LPS is a product of gram-negative bacteria and induces a T cell-independent stimulation of B cells mediated through Toll-like receptors (Browne 2012). LPS-stimulated B cells undergo polyclonal activation, which is unrestricted and non-specific, and result in the release of non-specific antibodies that target many antigens including microorganism related antigens (Montes et al. 2007; Xu et al. 2008; Bryan et al. 2010). We recently demonstrated that Taz deficiency in mice resulted in severe impairment in the immune function of LPS-activated B cells (Zegallai et al. 2021). In that study, LPS-activated TazKD B cells exhibited reduced glycolytic activity, mitochondrial oxidative phosphorylation, impaired proliferation and differentiation. In addition, the reduction in TazKD B cells metabolic activity was associated with a reduced expression of immunogenic markers and IgM secretion. Thus, TazKD B cells exhibit a deficiency in the T cell-independent activation pathway which could potentially be linked to the susceptibility of BTHS patients to serious infections. However, it was unknown

if Taz deficiency in B cells impaired B cell activation through T-cell dependent signaling mechanisms.

CD40 is a member of the tumor necrosis factor receptor superfamily and is expressed in many immune cells including B cells (Elgueta et al. 2009). CD40-stimulated B cells are crucial for immunological alerting of T cells (Possamaï et al. 2021). CD40 stimulated-B cells become potent antigen presenting cells capable of activating $CD4^+$ and $CD8^+$ T cells and induce an adaptive immunity that is specific and effective (Possamaï et al. 2021). Furthermore, CD40-stimulated B cells exhibit upregulated levels of co-stimulatory factors such as CD86 and CD69 (Elgueta et al. 2009; Possamaï et al. 2021). The stimulation of B cells with CD40 induces differentiation of B cells into long-lived plasma cells and memory B cells (D'Souza et al. 2017; Ise et al. 2018). In addition, CD40 stimulated B cells play an important role in the "T cell help" and anti-tumor immunity (Elgueta et al. 2009; Ara et al. 2018; Possamaï et al. 2021). It was recently demonstrated that $CD8^+$ T cell immunity was impaired in BTHS patients indicating the presence of poor T cell-dependent immunity in these patients (Corrado et al. 2020). In the current study, we examined the impact of Taz deficiency on T cell-dependent B cell activation. Our findings revealed that anti-CD40 + IL-4 stimulated TazKD B cells expressed lower levels of the co-stimulatory surface markers CD86 and CD69 in comparison to CD40 + IL-4 stimulated WT B cells. CD86 or (B7-2) is essential for programming B cells to be an antigen presenting cells thus enabling them to stimulate T cells (Zhang et al. 2004; Rau et al. 2009). CD69 is type II C-type Lectin that is expressed on the surface of many activated immune cells including B cells (Vazquez et al. 2009; Shinoda et al. 2012). The expression of CD69 on the surface of B cells stimulates the secretion of many cytokines and migratory factors for the recruitment of T cells (Vazquez et al. 2009; Maddur et al. 2014). Further, CD69 expression on B cells is crucial to induce T cell differentiation into T-helper cells (TH1 and TH-17) and T-regulatory cells (Tregs) (Vazquez et al. 2009; Maddur et al. 2014). Thus, the expression of

CD69 on the surface of immune cells, such as B cells, is necessary for the metabolic reprogramming of these cells (Cibrián and Sánchez-Madrid 2017). In addition, CD69 is important for promoting survival and differentiation of immune cells by activating the PI3K/mTOR signaling pathway (Soond et al. 2010). Our findings revealed that TazKD B cells stimulated by anti-CD40 + IL-4 exhibited a reduced percentage of cells in the S/G2 phase, with a higher percentage of cells in G0/G1 compared to WT B cells. In addition, the percentage of B cells undergoing apoptosis (sub G1 phase) were higher in anti-CD40 + IL-4 stimulated TazKD B cells compared to anti-CD40+IL-4 stimulated WT B cells. Thus, it is possible that the lowered expression of CD86 and CD69 coupled with a reduced cellular proliferation of anti-CD40 + IL-4 stimulated TazKD B cells might impair their ability to function as antigen presenting cells required for activation of T cells in BTHS patients.

Activated B cells require glycolytic-dependent metabolic reprogramming to support antibody production (Caro-Maldonado et al. 2014). We observed reduced glycolytic function in anti-CD40 + IL-4 stimulated TazKD B cells compared to anti-CD40+IL-4 stimulated WT B cells. In addition, IgM and IgG secretion into the medium was reduced in anti-CD40 + IL-4 stimulated TazKD B cells compared to anti-CD40+IL-4 stimulated WT B cells. The significance of reduced antibody production in anti-CD40 and IL-4 stimulated TazKD B cells is unknown as there is no evidence to suggest that BTHS patients exhibit impaired antibody production.

The 26S proteasome and the immunoproteasome are required to convert naïve B cells to antigen presenting cells. The 26S proteasome is an essential compartment for immune cells to degrade and eliminate damaged mitochondrial proteins and thus maintain intact mitochondrial function (Ibañez-Vega et al. 2019; Krämer et al. 2021). The immunoproteasome is a specialized type of proteasome required to process the antigenic peptides to be presented on the surface of antigen presenting immune cells (Santos et al. 2017; Abu-El-Rub et al. 2020).

We observed that both 26S proteasome activity and immunoproteasome activity was reduced in anti-CD40 +IL-4 stimulated TazKD B cells compared to anti-CD40 +IL-4 stimulated WT B cells. In addition, mitochondrial OXPHOS was markedly impaired in anti-CD40 + IL-4 stimulated TazKD B cells compared to anti-CD40 +IL-4 stimulated WT B cells. The decrease in these proteolytic activities in anti-CD40 + IL-4 stimulated TazKD B cells may contribute to observed mitochondrial dysfunction in these B cells.

In summary, we have observed that Taz deficiency in murine B cells results in a reduced ability of these cells to initiate T cell-dependent signaling. It has been reported that Taz deficiency in BTHS patients inhibit the T cell recruitment and activation. Our findings may provide an explanation for the poor immunity of T cells in BTHS patients through an inability of Taz deficient B cells to initiate T cell-dependent immune activation. It is possible that impaired T cell-dependent immunity in BTHS may increase patient susceptibility to endogenous pathogens. The findings in the current study complement our previous observation that Taz deficiency in B cells not only disrupts T cell-independent, but also T cell-dependent immunity. Understanding of the impact of Taz deficiency in the immune cells of BTHS patients may aid in the development of therapeutic strategies for treatment of infections in these patients.

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Conflict of interests

The authors of this study have no financial and non-financial conflict of interest.

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Ethical Approval

This study was performed with approval of the University of Manitoba Animal Policy and Welfare Committee which adheres to the principles for biomedical research involving animals developed by the Canadian Council on Animal Care and the Council for International Organizations of Medical Sciences and is reported in accordance with ARRIVE guidelines.

Informed Consent

Not applicable

Author contributions

AJM, HMZ, EA-E-R, GMH were responsible for conceptual design. HMZ, G.C.S. LKC, EMM performed the experiments. HMZ, EA-E-R analyzed the data. HMZ, EA-E-R and GMH wrote the manuscript. All authors have read and approved the final version of the manuscript.

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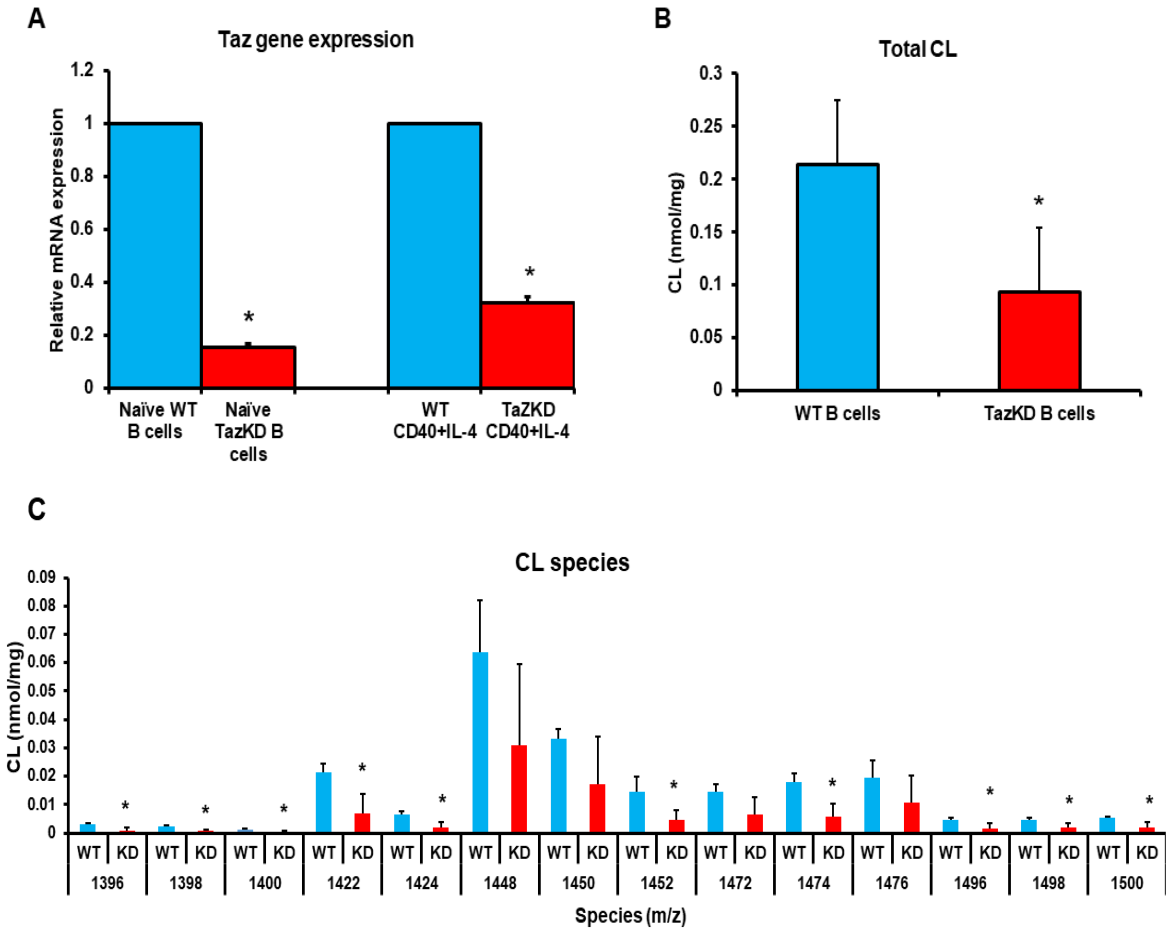
Supplementary Figure 1



Supplementary Figure 1. Tafazzin deficiency reduces total CL and fatty acyl molecular species in TazKD MSCs. A. Taz mRNA expression in WT and TazKD B cells. **B.** Total CL in WT and TazKD B cells. **C.** Fatty acyl species CL in WT and TazKD B cells. n=3. *p<0.05 compared with WT B cells.

CHAPTER VI. Tafazzin deficiency in mouse mesenchymal stem cells promote reprogramming of activated B lymphocytes toward immunosuppressive phenotypes

Rationale: In chapters III and IV, we determined that the Taz protein is required for proper B cells metabolic activities, proliferation, and function. Our studies revealed a novel role of the Taz protein in B lymphocytes function and further suggested that dysfunction of B cells may contribute to immunodeficiency in BTHS patients. In this chapter, we studied the impact of Taz deficiency on the immunosuppressive properties of MSCs. MSCs are important immunoregulatory cells and inhibit the activity of immune cells including B cells. Therefore, we also assessed the immunosuppressive effect of TazKD MSCs on WT B cells proliferation, function, and phenotypes.

Hypothesis: Taz deficiency enhances the immunosuppressive effect of MSCs on B lymphocytes immune function.

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Tafazzin deficiency in mouse mesenchymal stem cells promote reprogramming of activated B lymphocytes toward immunosuppressive phenotypes

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6.1 Abstract

Barth Syndrome (BTHS) is a rare X-linked genetic disorder caused by mutation in the TAFAZZIN gene. Tafazzin (Taz) deficiency in BTHS patients results in an increased risk of infections. Mesenchymal stem cells (MSCs) are well known for their immune-inhibitory function. We examined how Taz-deficiency in murine MSCs impact their ability to modulate the function of lipopolysaccharide (LPS)-activated wild type (WT) B lymphocytes. MSCs from tafazzin knockdown (TazKD) mice exhibited a reduction in mitochondrial cardiolipin compared to wild type (WT) MSCs. However, mitochondrial bioenergetics and membrane potential were unaltered. In contrast, TazKD MSCs exhibited increased reactive oxygen species generation and increased glycolysis. The increased glycolysis was associated with an elevated proliferation, phosphatidylinositol-3-kinase expression and expression of the immunosuppressive markers indoleamine-2,3-dioxygenase, cytotoxic T-lymphocyte-associated protein 4, interleukin-10, and cluster of differentiation 59 compared to controls. Blocking glycolysis with 2-deoxyglucose attenuated the TazKD-mediated increased expression of cytotoxic T-lymphocyte-associated protein 4 and interleukin-10. When co-cultured with LPS-activated WT B cells, TazKD MSCs inhibited B cell proliferation and growth rate and reduced B cell secretion of immunoglobulin M compared to controls. In addition, co-culture of LPS-activated WT B cells with TazKD MSCs promoted B cell differentiation toward interleukin-10 secreting plasma cells and B regulatory cells compared to controls. The results indicate that Taz deficiency in MSCs promote reprogramming of activated B lymphocytes toward immunosuppressive phenotypes.

Keywords: Barth syndrome, tafazzin, mesenchymal stem cells, activated B lymphocytes, immune function, mitochondria, cardiolipin, lipopolysaccharide

6.2 Introduction

Barth syndrome (BTHS) is a rare X-linked genetic disorder caused by mutations in the TAFAZZIN gene localized on chromosome Xq28 (1, 2). The product of the TAFAZZIN gene is the tafazzin (Taz) protein, a phospholipid transacylase enzyme that plays a critical role in maturation of the inner mitochondrial membrane phospholipid cardiolipin (CL) and remodels nascent CL to mature CL (3). CL comprises approximately 15-20% of the inner mitochondrial membrane phospholipid mass and is required for the activation of many enzymes and proteins involved in mitochondrial function (4, 5). BTHS patients exhibit CL deficiency and abnormal CL acyl chain composition leading to impairment and inhibition of ATP synthesis from oxidative phosphorylation (6). BTHS is characterized by cardiomyopathy, skeletal myopathy, neutropenia, and growth retardation (7). Cardiomyopathy is the main cause of mortality in BTHS patients. However, BTHS patients are vulnerable to severe infections as approximately 70% of patients develop neutropenia (2). The mechanism for the neutropenia in BTHS is not fully understood. In addition, there is little information of the impact of Taz deficiency on other cells of the immune system how other cells of the immune system might contribute to immunodeficiency in BTHS patients remains unclear.

Preclinical and clinical studies indicate that mesenchymal stem cells (MSCs) are among the best stem cells for treating many degenerative and chronic disorders (8). They have gained widespread attention due to their broad therapeutic applications and can be easily isolated from many sources including bone marrow, adipose tissues, umbilical cord and others (9). MSCs modulate and regulate many cells of the immune system through modification of their activity and differentiation portal (10). B cells are crucial for humoral immunity of the adaptive immune system (11). Altered immune function of B cells is observed in autoimmune diseases where B cell activation is uncontrolled and exaggerated or in immunodeficiency diseases where B cells

are poorly activated and recruited (12, 13). The recruitment, activation, differentiation and maturation of B cells is a complicated process and requires tight control. Activated B cells secrete antibodies, such as immunoglobulin-M (IgM) and immunoglobulin-G (IgG), and cytokines, such as interleukin-10 (IL-10) (14). The presence of MSCs at the site of B cell activation and maturation modulate B cell function and differentiation. MSCs prevent B cell proliferation and promote their arrest at the G0/G1 phase of the cell cycle and they secrete a cocktail of cytokines, including IL-10, transforming growth factor-β, prostaglandin E_2 and indoleamine 2,3, dioxygenase (IDO), which inhibit B cell terminal differentiation (15, 16). In addition, MSCs promote differentiation of B cells into B regulatory cells (Bregs) that induce an immune-tolerance state and impede differentiation of B cells into plasma cells and as a result suppress immunoglobulin secretion (15). Thus, the immunosuppressive properties of MSCs are important in regulating and tightly controlling the function of the immune system. However, the immune-inhibitory role of MSCs can be modulated and enhanced by genetic modifications which affect the function of other immune cells and may lead to an immunodeficiency state. To date no studies have examined how Taz deficiency in MSCs impacts the function of other cells of the immune system. In the current study, we demonstrate for the first time that Taz deficiency in murine MSCs regulates murine B cell function. Our results provide novel mechanistic information on the role of Taz in regulating immune system function.

6.3 Materials and Methods

Experimental animals

All experimental procedures in this study were performed with approval of the University of Manitoba Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines and regulations. This study is reported in accordance with ARRIVE

guidelines. All animals were housed in pathogen-free facility (12 h light/dark cycle) at the University of Manitoba. Taz knockdown (TazKD) mice were generated by mating transgenic male mice containing doxycycline (dox) inducible Taz specific short-hair-pin RNA (shRNA) (B6.Cg-Gt(ROSA)26Sortm1(H1/tetO-RNAi:Taz,CAG-tetR)Bsf/ZkhuJwith female C57BL/6J mice from Jackson Laboratory (Boston,MA). Taz knock down was maintained postnatally by administering dox (625 mg of dox/kg of chow) as part of the rodent chow (Rodent diet TD.01306 from Harlan, Indianapolis, USA) as described previously (17). Male mice were used for the experiments between 10 and 14 weeks of age. Male mice positive for the Taz shRNA transgene were identified by PCR as described previously (17).

MSCs isolation and culture

Mice MSCs were isolated from the femurs and tibias by flushing the bone marrow cells (18). The connective tissue around the bones was removed, then the bone marrow plugs were flushed using Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum (FBS), 100 units/ml penicillin G, and 0.1 mg/ml streptomycin. The bone cavities were repeatedly flushed to obtain enough marrow cells. Then, the cells were plated and cultured in the same media at 37 °C with 5% CO_2. The medium was changed in the next day and the non-adherent cells were removed. Every 3 days, medium was replaced and the cells were cultured until confluency exceeded 90%. Morphology of MSCs was determined by taking phase contrast images using Cytation 5 imaging reader (BioTek, USA). In some experiments, MSCs were incubated with 1mM 2-deoxyglucose (2DG) for 24 h to block glycolysis and the levels of IL-10 and CTLA-4 determined using ELISA kits. Glycolysis Assay (Extracellular acidification) (Catalog no. ab197244, abcam). Mouse IL-10 DuoSet ELISA (Catalog no. DY417-05) was obtained from R&D Systems (Minneapolis, MN). CTLA-4 Mouse ELISA Kit (Catalog no. **EMCTLA4)** was obtained from Thermo Fisher. Unless otherwise indicated, all other reagents

used were of analytical grade and were obtained from either Thermo Fisher Scientific (Winnipeg, MB) or Sigma-Aldrich (Oakville, ON).

B Cells isolation and co-culture of MSCs and B cells

Splenic naïve B lymphocytes were isolated from WT mice by magnetic bead negative selection using EasySep Mouse B Cell Isolation Kit (Catalog no. 19854) from STEMCELL Technologies (Vancouver, BC). B cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% antibiotic/antimycotic, and 2-mercaptoethanol at 37°C with 5% CO_2. Subsequent to isolation B cells were stimulated with 10 µg/ml lipopolysaccharide (LPS) (Catalog no. L2887) from Sigma-Aldrich (Oakville, ON). For co-culture with MSCs, the naïve B cells were stimulated by LPS (10 µg/ml) for 24 h then co-cultured with WT MSCs or TazKD MSCs for 72 h in 24-well plates at ratio 1:10 of MSCs:B cells. In these experiments, the LPS was washed away with phosphate buffer saline prior to co-culture with MSCs.

Metabolic assays

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured in MSCs using a Seahorse XF24 Extracellular Flux Analyzer and kits from Agilent Technologies (Santa Clara, CA). Briefly, isolated WT and TazKD MSCs were seeded in XF24 24-well plates coated at density of 4×10^5 cells per well. For mitochondrial function stress test (OCR assay) the following final concentrations of drugs were used: 1 µM oligomycin, 1 µM FCCP, 1 µM antimycin A, and 1 µM rotenone as per the manufacturer's instructions. The OCR values of the basal respiration, maximal respiration, and spare respiratory capacity were determined to assess the mitochondrial function. For glycolytic function test (ECAR assay), 10 µM glucose, 1 µM oligomycin, and 25 µM 2DG were used as per the manufacturer's instructions. The extracellular acidification rates (ECAR) for glycolysis, glycolytic capacity,

and glycolytic reserve were determined. At the end of the assay, all measurements were normalized to µg of protein in each well.

Measurement of Mitochondrial Membrane Potential.

WT MSCs and TazKD MSCs were seeded onto 96-well plate until they attained 80% confluence. The mitochondrial membrane potential was assessed using a TMRE mitochondrial membrane potential assay kit (Catalog #: K238. BioVision) according to the manufacturer's protocol. The fluorescence intensity was read at excitation=549nm and emission=575nm. The images were taken by Cytation 5 imaging reader (BioTek, USA) at 100 magnification scale.

Measurement of Mitochondrial ROS by using MitoSOX staining.

WT MSCs and TazKD MSCs were incubated with 5 µM of the mitochondrial-specific superoxide indicator MitoSOX (Catalog no. A36006, ThermoFisher) for 20 min. at 37ºC in dark. Then, the cells were washed with Hank's balanced salt solution containing calcium and magnesium (HBSS, Catalog no. 14025-092, Gibco) twice and re-suspended in HBSS. The fluorescence was measured at 568 nm excitation and 581 nm emission.

MSCs and B cells proliferation assay

MSCs and B cells proliferation were measured using the cell proliferation assay kit (CellTiter 96 AQ_{ueous}, Promega) according to manufacturer's instructions. Cells were cultured and treated with Cell Titer 96 aqueous solution. Then, the absorbance was recorded at 490 nm.

Western blot analysis

Cell proteins were extracted using lysis buffer (RIPA Lysis buffer, catalog no. 89900) from Thermo Fisher (Winnipeg, MB). The total protein levels were measured by the Bradford method (19). Briefly, 30 µg of protein was mixed with an equal volume of 2×Laemmli buffer

supplemented with 5% 2-mercaptoethanol (Catalog no. 1610737) then loaded in 10% polyacrylamide precast gel (TGX™ FastCast™ Acrylamide Starter Kit, 10% catalog no. 1610172) from BioRad Laboratories (Mississauga, ON). After 1 h electrophoresis, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane at 4 °C. This was followed by block the membrane using 5% milk in a solution of TBS containing 0.5% Tween 20 (TBS-T) at room temperature for 1 h. Then, the membrane was washed with TBS-T three times and incubated with primary antibody an overnight at 4°C. The primary antibody was removed next day and the membrane was incubated with secondary antibody at room temperature for 1 h. ECL substrate (Clarity Western ECL Substrate, Catalog no.1705060) from BioRad Laboratories (Mississauga, ON) was used to visualize the protein bands. The primary antibodies used in for Western blot analysis were as follows: PI 3-kinase p85α (Catalog no. sc-1637), IDO (Catalog no. sc-137012), CTLA-4 (Catalog no. sc-376016), IL-10 (Catalog no. sc-365858), CD59 (Catalog no. sc-59095), Tafazzin (Catalog no. sc-365810), and β-Actin (Catalog no. sc-47778) from Santa Cruz Biotechnology (Dallas, TX). Band intensity was quantified using ImageJ software.

B cells population doubling and % of growth rate determination

The naïve WT B cells were stimulated with 10 µg/ml LPS for 24 h, then the activated B cells were co-cultured with WT MSCs or TazKD MSCs for 72 h at ratio of 1:10 (MSCs:B cells) as above. After 72 h of coculture B cells were isolated as described below and stained with Trypan blue and the number of live B cells determined. The doubling time was calculated using the following formula: Doubling time = time of culture × log (2)/log (final cell number) – log (initial cell number). The percent growth rate was calculated as follows: The percent change from one period to another was determined using the following formula: Percent Change = 100

× (final cell number–initial cell number)/initial cell number. The percent growth rate was then determined using the following formula: Percent Growth Rate = Percent Change /Time (h).

Measurement of antibodies and IL-10 concentration

After stimulation of naïve WT B cells with 10 μg/ml LPS for 24 h, the activated B cells were co-cultured with WT MSCs or TazKD MSCs for 72 h. The supernatant fractions were collected to measure the antibodies and IL-10 concentrations. IgG, IgM, and IL-10 levels in cell culture supernatants were measured using isotype-specific enzyme-linked immunosorbent assay (ELISA) kits. IgG (total) mouse uncoated ELISA kit (Catalog no.88-50400-22) and IgM mouse uncoated ELISA kit (Catalog no.88-50470-22) were from Thermo Fisher (Winnipeg, MB). Mouse IL-10 DuoSet ELISA (Catalog no. DY417-05) was obtained from R&D Systems (Minneapolis, MN).

Flow cytometry analysis

LPS activated B cells were co-cultured with WT MSCs or TazKD MSCs at ratio 1:10 (MSCs: B cells). The surface markers of B cells were assessed after co-culture with MSCs by flow cytometry analysis. Briefly, after 72 h of co-culture, B cells in the supernatant were collected and centrifuged at 1200 rpm for 5 min. Then, the pellet was washed 2 times with PBS and suspended in 500 μl FACS buffer (PBS containing 2% FBS). Plasma cells were analyzed by staining with fluorochrome-conjugated antibodies against B220, CD19, and CD138 (BioLegend, San Diego, CA). IL-10 producing B cells and Plasma cells were analysed via intracellular cytokines staining described below. The cells were stimulated for 4 h with 50 ng/mL phorbol myristic acetate 500 ng/mL ionomycin, and 10 μg/mL brefeldin A. Cells were fixed, surface stained for B220 and CD138, and subsequently stained for intracellular IL-10 (BD Biosciences, Mississauga, ON). Cells were then washed with FACS buffer then were

acquired and analyzed using the BD FACS Canto II cytometer and FlowJo software (BD Biosciences), respectively. Cells were gated first on lymphocytes, single cells by forward scatter width, and then dead cells gated out using Aqua viability dye (BioLegend, San Diego, CA). Samples were then labeled with specific antibodies. Anti-CD19 (APC-Cy7), anti-B220 (PE-Cy7), anti-CD138 (APC), anti-IgD (FITC), anti-IL-10 (PE), anti-Sca-1 (BV421), anti-IgM (PE-Cy7), anti-CD5 (PE) were from BD Biosciences. Cells were analyzed by flow cytometry using a BD FACS Canto II instrument. FlowJo software was used for data analyses.

Lipid Mass Spectrometry

Electrospray ionization - mass spectrometry coupled to high performance liquid chromatography was performed to quantify CL mass and fatty acyl molecular species as previously described (20).

Statistical analysis

All experimental results were expressed as mean ± SD. The p values were calculated using two tailed unpaired Student's t test unless otherwise specified/one-way-analysis of variance followed by multiple comparisons by Tukey's test. A p value of < 0.05 was considered statistically significant.

6.4 Results

Taz deficiency in MSCs increases glycolysis but does not alter mitochondrial function

Taz deficiency results in decrease of CL (21). Initially we examined Taz expression and CL levels in MSCs. TazKD MSCs exhibited a 55% ($p<0.0001$) reduction in Taz protein expression associated with a 42% ($p=0.005$) reduction in CL compared to WT MSCs (Supplementary Fig. 1A &1B). The reduction in CL was seen across all CL molecular species examined

(Supplementary Fig. 1C). Morphology of TazKD MSCs were identical to that of *WT MSCs (Supplementary Fig. 2).* We next assessed mitochondrial respiration in WT MSCs and TazKD MSCs. Surprisingly, we observed no alteration in mitochondrial oxygen consumption rate (OCR) including basal respiration (measured before addition of inhibitors), maximal respiration (determined after addition of FCCP), or spare respiratory capacity (the difference between the maximal OCR and basal OCR) in TazKD MSCs compared to WT MSCs (Fig. 1A). Thus, mitochondrial respiration did not appear to be impacted by Taz deficiency and CL loss in MSCs. We then measured extracellular acidification rate (ECAR) in wild type MSCs and TazKD MSCs. TazKD MSCs exhibited about 40% increase (p =0.03) in glycolysis compared to WT MSCs (Fig. 1B). In contrast, glycolytic capacity and glycolytic reserve were not altered in TazKD MSCs (Fig. 1B). Since our metabolic analysis indicated that mitochondrial function was not altered in TazKD MSCs, we investigated whether the mitochondrial membrane potential was affected by Taz deficiency. TMRE (tetramethylrhodamine, ethyl ester) is a positively charged red-orange fluorescent dye that accumulates in active negatively charged mitochondria. Therefore, decreased fluorescent intensity indicates the presence of inactive mitochondria. WT MSCs and TazKD MSCs were incubated with TMRE and fluorescent intensity determined. No change in fluorescent intensity was observed between WT MSCs and TazKD MSCs confirming that the mitochondrial activity was unaltered in TazKD MSCs (Fig. 1C). Previous studies have shown that Taz deficiency in cell models of BTHS increases reactive oxygen species (ROS) generation (22, 23). Interestingly, although TazKD MSCs exhibited an 20% increase in ROS generation compared to WT MSCs (Supplementary Fig. 3) this did not seem to impair mitochondrial OCR. Thus, TazKD MSCs exhibit an increase in ROS generation and glycolysis. In MSCs increased glycolysis is associated with a shift in their immunological properties (24). This interesting finding prompted us to investigate the immunomodulation abilities of TazKD MSCs.

Figure 1

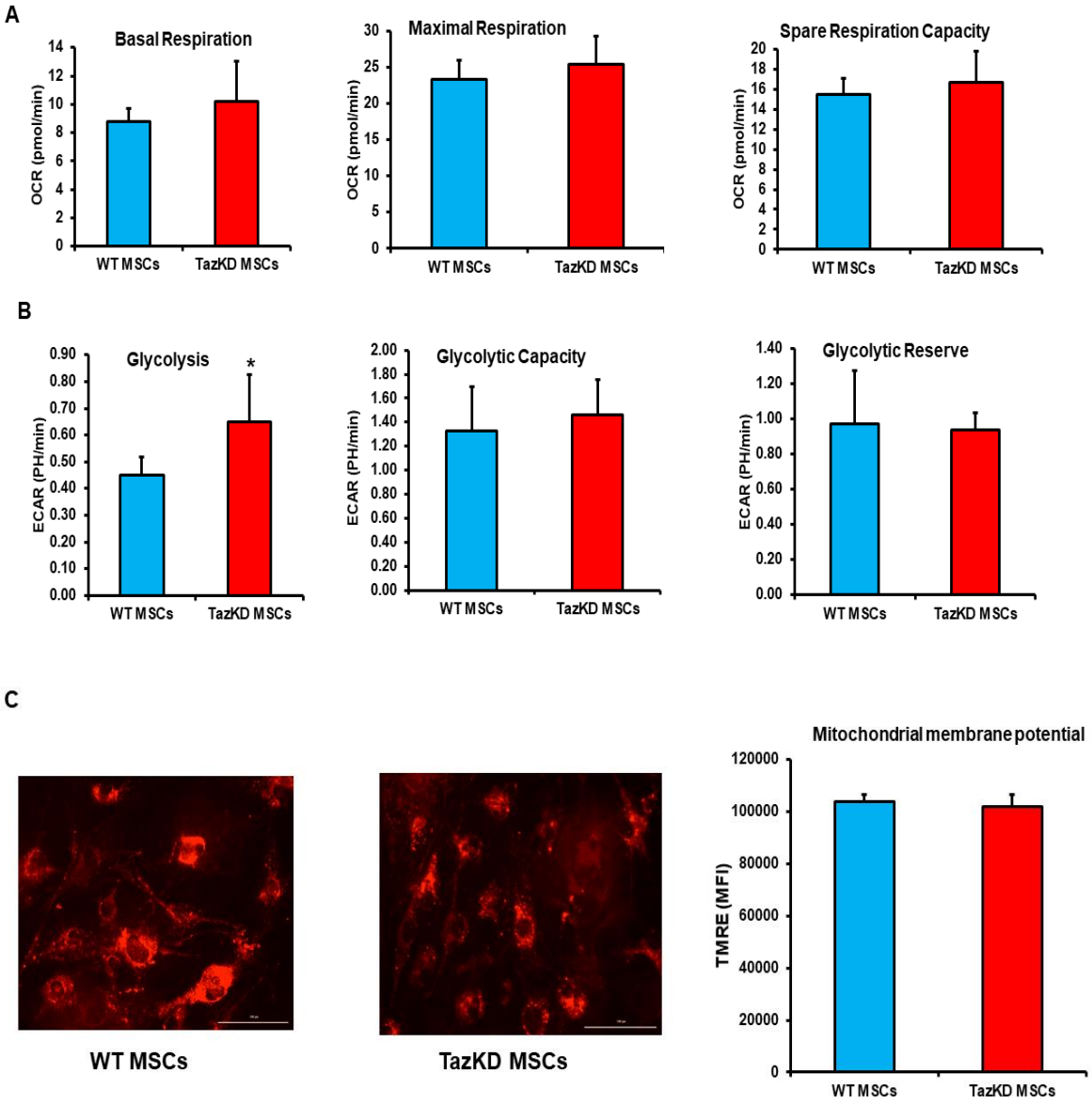


Figure 1. TazKD MSCs exhibit increased glycolysis. Mitochondrial function and glycolytic activity were analyzed in WT MSCs and TazKD MSCs as described in Materials and Methods. **A**. Basal respiration, maximal respiration and spare respiratory capacity. **B**. Glycolysis, glycolytic capacity and glycolytic reserve. **C.** Mitochondrial membrane potential in WT and TazKD MSCs was assessed by using TMRE Assay as described in Materials and Methods. The images were taken by Cytation 5 imaging reader at 100 magnification scale. n=4. *p<0.05 compared with WT MSCs cells.

Tafazzin deficiency promotes MSCs proliferation and increases expression of immunosuppressive markers

Initially we examined the impact of Taz deficiency on MSCs proliferation. We found that the proliferation rate of TazKD MSCs was increased 27% (p=0.009) compared to WT MSCs (Fig. 2A). The phosphoinositide-3-kinase (PI3K) pathway is essential for MSCs survival and proliferation and upregulation in PI3K expression is considered a marker for increased proliferation of MSCs (25). We thus examined mRNA and protein expression of PI3K in TazKD MSCs. We found that PI3K protein expression was elevated 78% (p=0.04) in TazKD MSCs compared to WT MSCs (Fig. 2B).

MSCs are immunosuppressive cells which can modulate and suppress other cells of the immune system through expression of a variety of immunosuppressant molecules including IDO, cytotoxic T lymphocyte antigen 4 (CTLA-4), IL-10, and cluster of differentiation 59 (CD59) (16). These markers modify the function of many immune cells including B cells and mitigate their activation (26). We thus measured protein expression of the major immunosuppressive molecules IDO, CTLA-4, IL-10, and CD59. Interestingly, protein expression of all these immunosuppressive markers was markedly increased in TazKD MSCs compared to WT MSCs (Fig. 2C). IDO expression was increased 187% (p=0.008), CTLA-4 increased 89% (p=0.02), IL-10 increased 90% (p<0.0001) and CD59 increased 148% (p=0.001) in TazKD MSCs compared to WT MSCs. Thus, MSCs isolated from TazKD mice exhibit a higher proliferation rate and an elevated expression of immunosuppressive markers.

A previous study demonstrated that interferon-gamma treatment of human MSCs resulted in remodeling of MSC metabolic pathways toward glycolysis, which was required to sustain the secretion of immunosuppressive factors (24). Since our observed increased proliferation and expression of immunosuppressive markers was consistent with an increase in glycolysis in

TazKD MSCs, we determined if blocking glycolysis attenuated the elevated expression of immunosuppressive markers in TazKD MSCs. WT MSCs and TazKD MSCs were incubated minus or plus the glycolytic inhibitor 2DG and IL-10 and CTLA-4 levels examined. Incubation of TazKD MSCs with 2DG reduced the levels of glycolysis, IL-10 and CTLA-4 compared to TazKD MSCs (Supplementary Fig. 4A-C). Thus, the elevated glycolysis in TazKD MSCs was associated with increased expression of immunosuppressive markers.

Figure 2

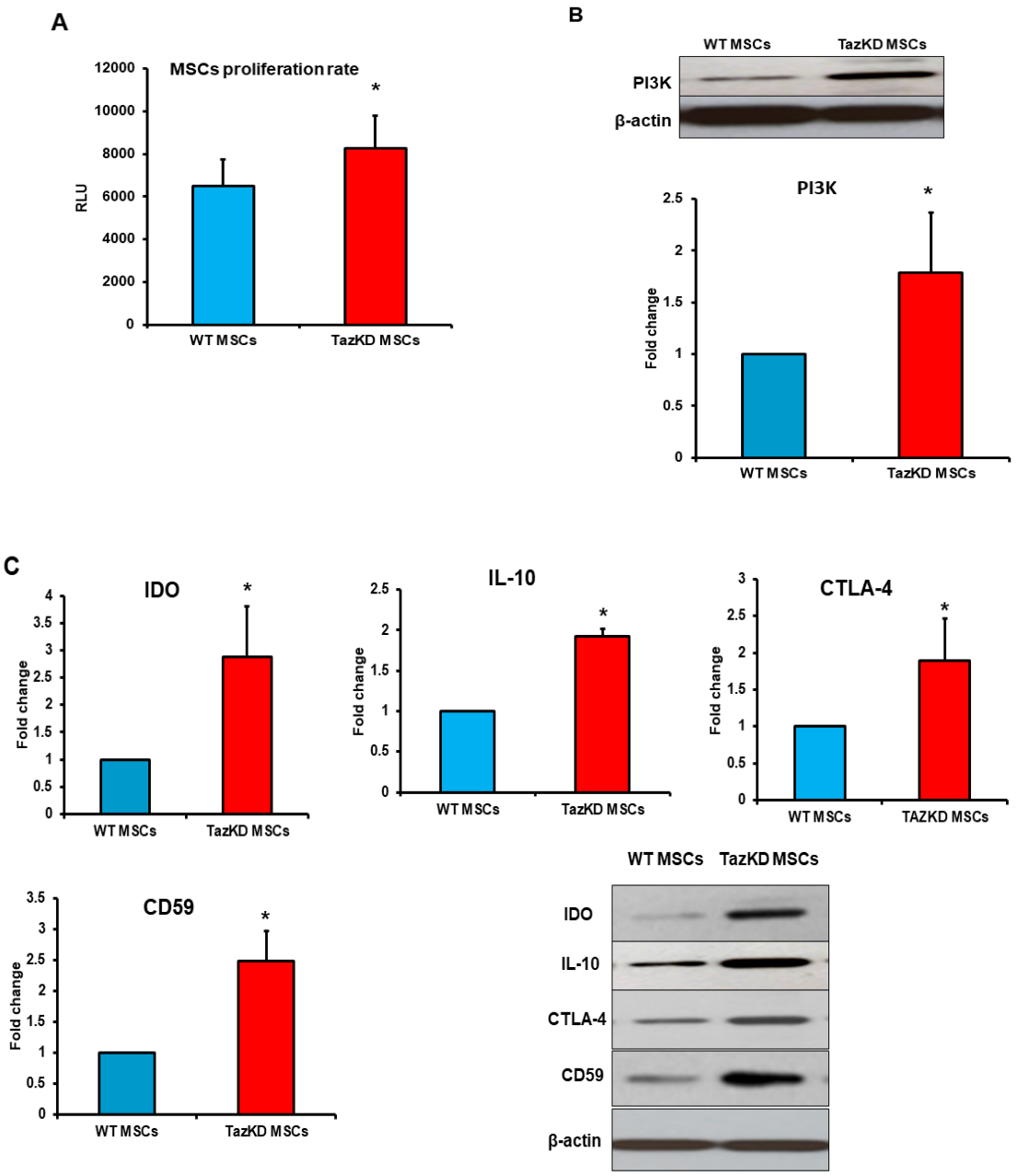


Figure 2. Tafazzin deficiency promotes MSCs proliferation and increases immunosuppressive markers. Proliferation rate, PI3K protein expression and protein levels of immunosuppressive markers in WT and TazKD MSCs were determined as described in Materials and Methods. **A.** Proliferation rate of WT and TazKD MSCs, **B.** PI3K protein expression in WT and TazKD MSCs. **C.** Protein levels of immunosuppressive markers IDO,

CTLA-4, IL-10, CD59, with β-actin as loading control in WT and TazKD MSCs. n=4. *p<0.05 compared with WT MSCs cells.

TazKD MSCs inhibit B cell proliferation and reduce the rate of B cell growth

B lymphocytes are indispensable for adaptive and humoral immunity and play a vital role in maintenance of normal immune response. Inhibition of B cell proliferation and differentiation weakens the immune system and contributes to immunodeficiency (27). Previous studies have shown that MSCs mitigate the proliferation of immune cells by either secreting immunosuppressive molecules or through cell-to-cell contact (28). We thus investigated the effect of TazKD MSCs on B cell proliferation. B cells were isolated from WT mice and activated by incubation with LPS for 24 h. LPS binds to Toll-like receptor-4 and promotes murine B proliferation (29). Subsequently, WT MSCs or TazKD MSCs were co-cultured with activated B cells for 72 h and B cell proliferation determined. B cell proliferation was inhibited 60% (p=0.0001) when co-cultured with TazKD MSCs compared to WT MSCs (Fig. 3A). In addition, co-culture of activated B cells with TazKD MSCs increased the population doubling time of B cells by 40% (p=0.003), and decreased B cell growth rate by 50% (p=0.002) compared to B cells co-cultured with WT MSCs (Fig. 3B, 3C). Thus, Taz deficiency in MSCs inhibits B cell proliferation and reduces the rate of B cell growth.

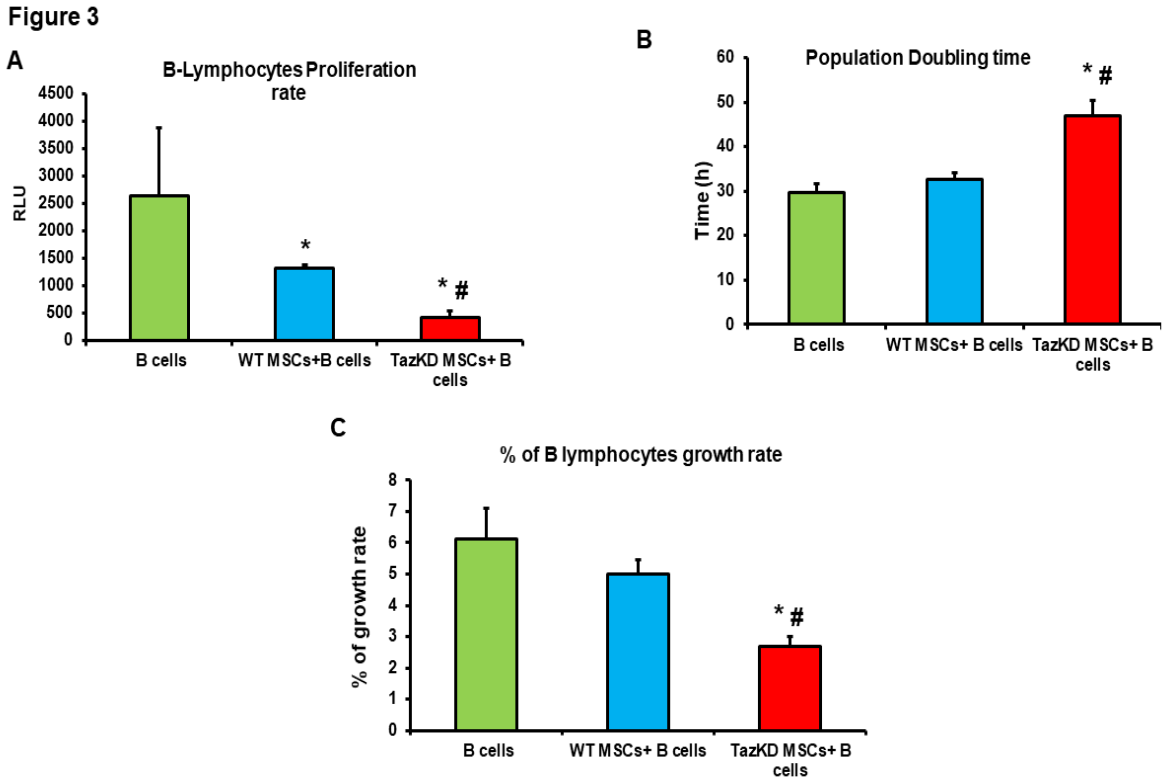


Figure 3. TazKD MSCs inhibit B cells proliferation and decrease B cell growth rate. WT B cells were activated with LPS then co-cultured with WT MSCs or TazKD MSCs and B cell proliferation rate, doubling time and percent growth were determined as described in Materials and Methods. **A.** B cells proliferation rate. **B.** B cells population doubling time, **C.** Percent B cell growth rate. n=3-5. *p< 0.05 compared with B cells. #p<0.05 compared with WT MSCs.

TazKD MSCs decrease IgM production and increase IL-10 secretion in B cells

Activation of murine B cells with LPS promotes synthesis and secretion of antibodies and MSCs have the ability to inhibit antibody secretion by LPS-stimulated B cells in co-culture (30). We investigated the effect of TazKD MSCs on B cells antibody secretion. LPS activated B cells were co-cultured with either WT MSCs or TazKD MSCs as above and IgG and IgM secretion into the medium determined. Co-culture of B cells with TazKD MSCs reduced medium IgM levels by 46% ($p<0.0001$) compared to B cells co-cultured with WT MSCs (Fig. 4A). Medium IgG concentrations were unaltered (Fig. 4B).

MSCs promote immune tolerance by inducing a regulatory B cells (Bregs) phenotype. Bregs suppress the immune response by secreting the anti-inflammatory cytokine IL-10 (31). LPS activated B cells were co-cultured with either WT MSCs or TazKD MSCs as above and medium IL-10 concentration determined. Co-culture of B cells with TazKD MSCs markedly increased IL-10 levels by 70% ($p=0.001$) compared to B cells co-cultured with WT MSCs (Fig. 4C). Thus, Taz deficiency in MSCs reduces IgM production and increases IL-10 secretion of B cells.

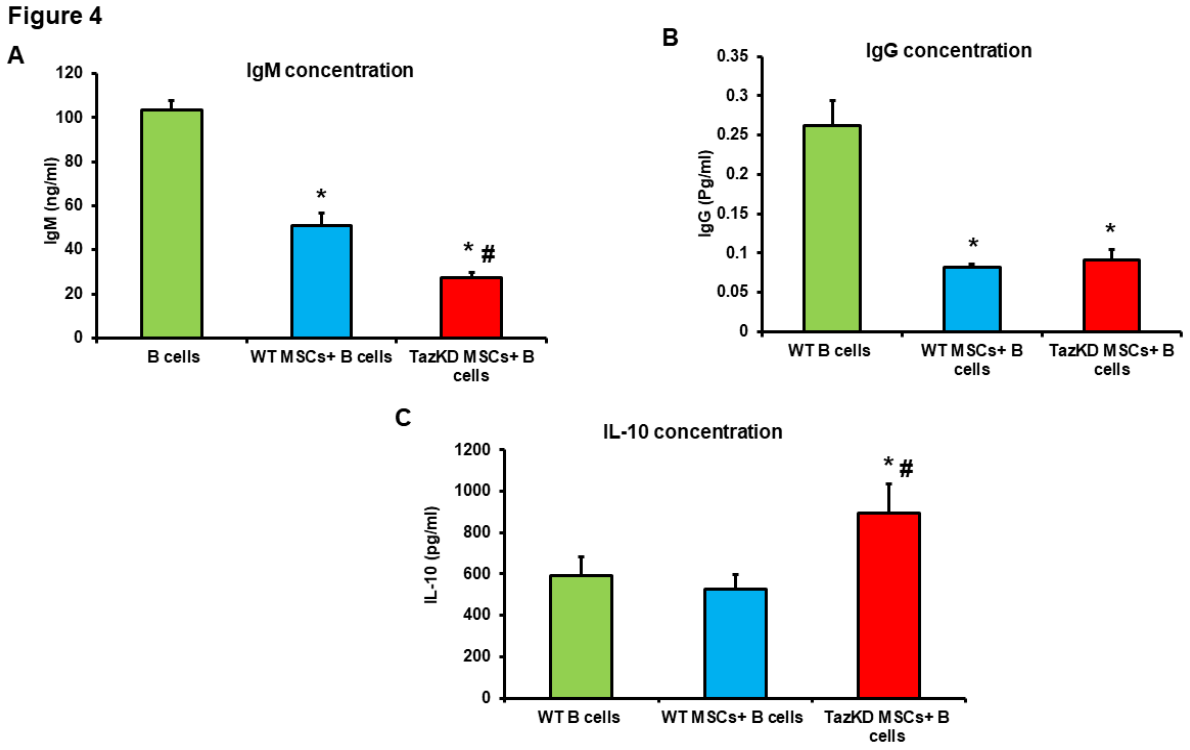


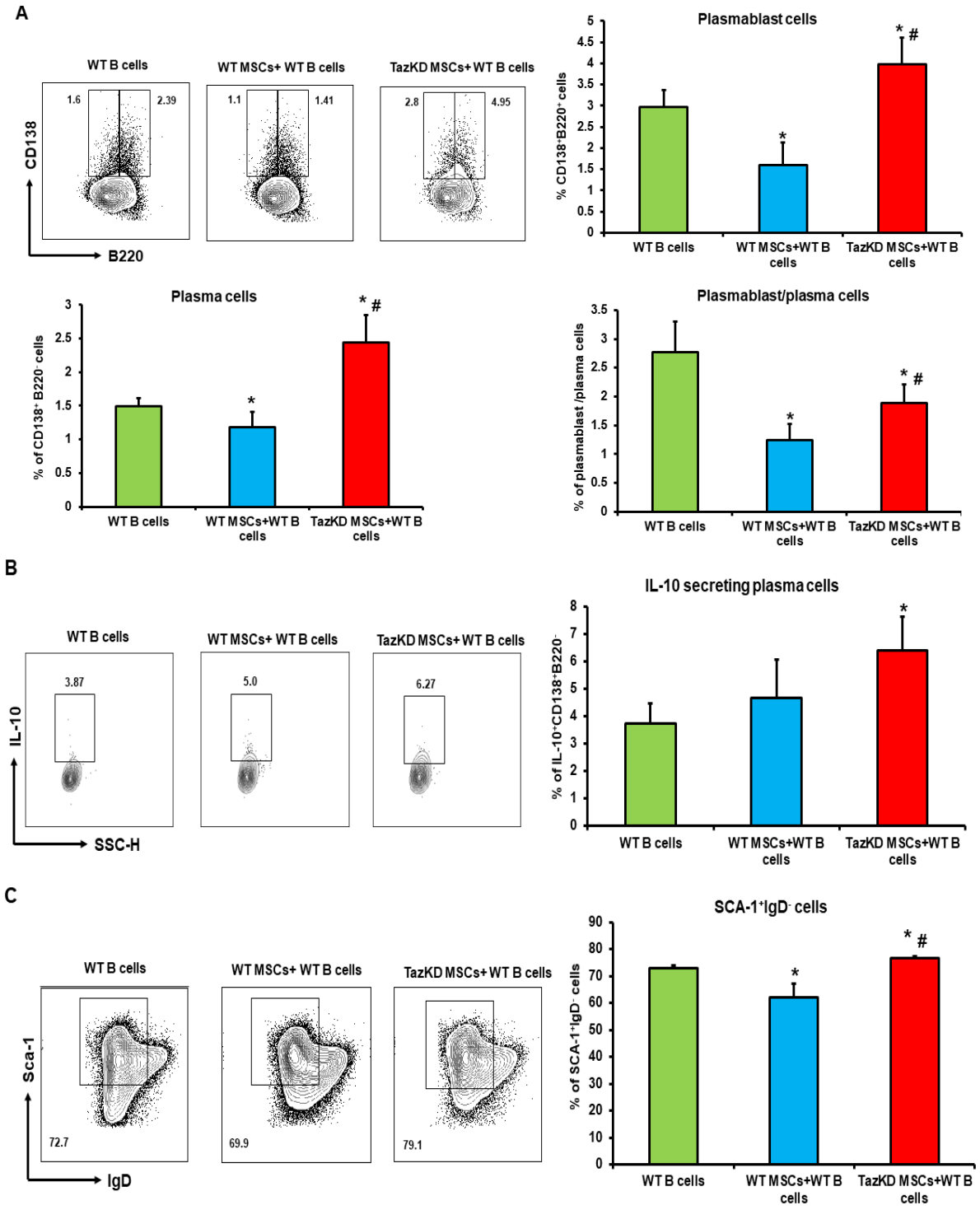
Figure 4. TazKD MSCs decrease IgM production and increase IL-10 secretion by B cells. WT B cells were activated with LPS then co-cultured with WT MSCs or TazKD MSCs and IgM, IgG and IL-10 levels determined as described in Materials and Methods. **A.** IgM, **B.** IgG and **C.** IL-10. n=3-5. *p<0.05 compared with B cells. #p<0.05 compared with WT MSCs cells.

TazKD MSCs induce the formation of B cell immunosuppressive phenotypes

B cells differentiate into antibody secreting cells known as plasma cells. Plasma cells play a pivotal role in providing sustained and long-time immunity against many infections (32). To determine if Taz deficiency impacted B cell differentiation into plasma cells and immunosuppressive phenotypes, LPS activated B cells were co-cultured with either WT MSCs or TazKD MSCs as above and flow cytometry analysis of $CD138^{+}/B222^{-}$ plasma cells, $CD138^{+}/B222^{+}$ plasmablasts, IL-10 secreting plasma cells, IL-10 secreting Bregs, and IL-10 secreting B cells were performed. The flow cytometry gating strategy for analysis of B cell subsets is shown in supplementary Fig. 5A & 5B. Co-culture of B cells with TazKD MSCs increased the percentage of undifferentiated plasmablasts by 100% ($p<0.01$) compared to B cells co-cultured with WT MSCs (Fig. 5A). Plasmablasts are an earlier stage of B cells differentiation that have the tendency to become a terminally non-dividing plasma cells. The differentiation of B cells into plasma cells increased by 100% ($p<0.001$) compared to B cells co-cultured with WT MSCs (Fig. 5A). Despite this increase in plasma cells percentage, the plasmablasts/plasma cells ratio was found to be higher by 40% ($p<0.05$) after co-culturing of B cells with TazKD MSCs compared to B cells co-cultured with WT MSCs (Fig. 5A). In addition, co-culture of B cells with TazKD MSCs increased the number of IL-10 secreting plasma cells compared to B cells co-cultured with WT MSCs (Fig. 5B). Immunoglobulin D (IgD) is known to signal B cell activation and its presence on B cells indicate a mature and functional B cell that can take part in immune defense (33, 34). Stem cell antigen 1 (Sca-1) is a known marker for hematopoietic progenitor cells and its expression is indicative of immature B cells (35, 36). Co-culture of TazKD MSCs with B cells increased the number of $Sca\text{-}1^{+}/IgD^{-}$ B cells by 20% ($p<0.01$) compared to B cells co-cultured with WT MSCs (Bregs Fig.5C). IL-10 secreting Bregs are a potent immunosuppressive subset of B cells. We found that co-culture of TazKD MSCs with B cells increased the population of IL-10 secreting by 30% ($p<0.05$)

compared to B cells co-cultured with WT MSCs (Fig. 5D). In addition, the number of IL-10 secreting B cells were increased 35% ($p<0.05$) in B cells co-cultured with TazKD MSCs compared to B cells co-cultured with WT MSCs (Fig. 5E). Thus, the presence of TazKD MSCs had the ability to switch the phenotype of B cells toward IL-10 secreting B cells which are a highly immunosuppressive form of B cells demonstrating that co-culture of activated B cells with TazKD MSCs induces the formation of B cell immunosuppressive phenotypes.

Figure 5


A
WT B cells
WT MSCs+ WT B cells
TazKD MSCs+ WT B cells
CD138
B220
1.6
2.39
1.1
1.41
2.8
4.95
Plasmablast cells
% CD138+B220+ cells
Plasma cells
% of CD138+ B220- cells
Plasmablast/plasma cells
% of plasmablast /plasma cells
B
IL-10 secreting plasma cells
% of IL-10+CD138+B220-
IL-10
SSC-H
3.87
5.0
6.27
C
SCA-1+IgD- cells
% of SCA-1+IgD- cells
Sca-1
IgD
72.7
69.9
79.1

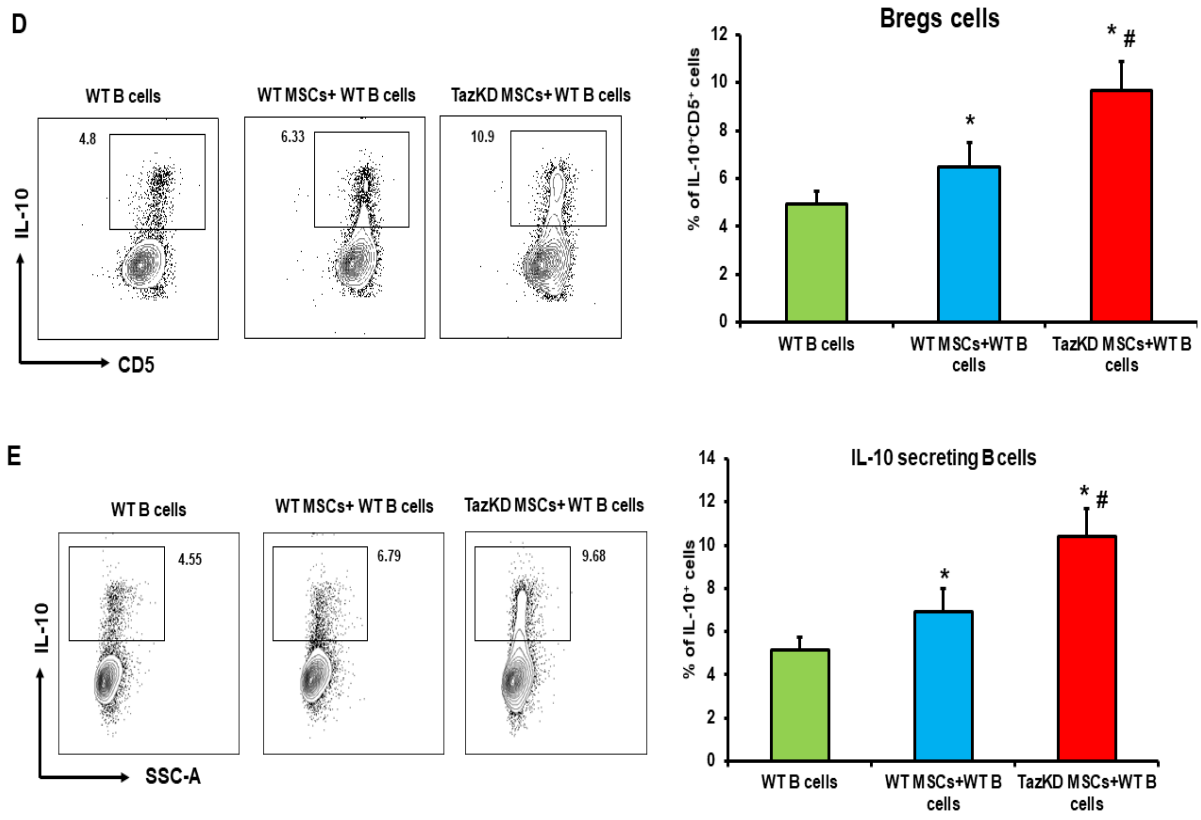


Figure 5. TazKD MSCs induce the formation of immunosuppressive B cell phenotypes. WT B cells were activated with LPS then co-cultured with WT MSCs or TazKD MSCs and B Cells collected from the supernatants after co-culture and stained with specific antibodies and subjected to flow cytometry analysis as described in Materials and Methods. Representative flow cytometry plots are shown. **A.** Percent of plasmablast cells, plasma cells, and plasmablast/plasma cells ratio in the different groups. **B.** Percent of IL-10 secreting plasma cells. **C.** Percent of Sca-1^{+}/IgD^{-} immature B cells. **D.** Percent of IL-10 secreting Bregs. **E.** Percent of IL-10 secreting B cells. n=4 *p<0.05 compared with B cells. #p<0.05 compared with WT MSCs cells.

6.5 Discussion

BTHS is a rare X-linked autosomal recessive disease where patients display cardiomyopathy, skeletal myopathy, growth retardation, neutropenia and susceptibility to infections. BTHS and its associated complications are related to abnormal mitochondrial function and oxidative phosphorylation due to CL deficiency as a result of mutations in the TAFAZZIN gene which encodes the Taz protein (37). Taz is a mitochondrial inner membrane CL transacylase required for CL remodeling and maturation. CL is an essential lipid of the inner mitochondrial membrane and provides vital role for the proper function of many enzymes and complexes involved in oxidative phosphorylation (38). Previous studies have reported that Taz deficiency in BTHS patients results in susceptibility to multiple and serious infections. Neutropenia has been observed in BTHS patients and this is proposed to be a link to the weaken innate immunity in these patients (2, 39). However, the role of Taz in other cells of the immune system is beginning to emerge. For example, Taz deficiency resulted in improper activation and differentiation of $CD8^+$ T cells (40). In addition, Taz deficiency in Mast cells impaired their exocytotic mechanisms (41). Moreover, a mild and consistent leukopenia was observed in mice transplanted with TAFAZZIN knockout fetal liver cells which manifested as a mild decrease across lineages, including peripheral B-cells, T-cells, myeloid cells, and neutrophils (42). B lymphocytes are essential for adaptive and humoral immunity. We recently reported that Taz deficiency in B cells reduced their metabolic activity and function during activation (43). However, the role of Taz in regulating MSCs function and the impact of Taz deficiency in MSCs on other cells of the immune system had never been investigated.

MSCs undergo an energy metabolism switch depending on the stress of the microenvironment or an alternation in their genetic and epigenetic characteristics (44). Mitochondrial membrane potential is a key determinant of mitochondrial activity and dynamics and it is maintained

during normal oxidative phosphorylation (45). Surprisingly, although Taz deficiency reduced CL in MSCs, it did not impact mitochondrial energy metabolism nor mitochondrial membrane potential. This is consistent with a previous study in which Taz knockdown did not disrupt mitochondrial morphology nor oxidative phosphorylation in hematopoietic stem and progenitor cells (46). Interestingly, TazKD MSCs exhibited an increase in ROS generation even in the presence of normal mitochondrial OCR. ROS can act as an activator of transcription factors and modulate their activity either directly or indirectly in MSCs (47). In addition, the accumulation of ROS may play an important signaling role in the metabolic reconfiguration of MSCs (24). It has been revealed that the oxidative stress triggers glycolysis as a defensive mechanism which may explain the high glycolysis level in TazKD MSCs (48).

Glycolysis is an anaerobic energy production mechanism extensively used by MSCs and increased glycolysis is associated with an increase in MSCs immunosuppressive abilities (24, 49). For example, remodeling of human MSCs metabolism towards glycolysis under interferon-gamma treatment potentiated stemness and immunomodulation through increased secretion of immunosuppressive markers (24). We observed that Taz deficiency in murine MSCs resulted in increased glycolysis and this was associated with increased PI3K mRNA and protein expression indicative of increased proliferation. In addition, the increased proliferation of TazKD MSCs was associated with an increase in the expression of the immunosuppressive markers IDO, CTLA-4, IL-10 and CD59. Moreover, blocking glycolysis through incubation of MSCs with 2DG attenuated the TazKD-mediated increase in expression of IL-10 and CTLA-4 suggesting that the impact of Taz deficiency in altering MSC metabolic function may contribute to the immunosuppressive phenotype.

IDO is a soluble immunosuppressive factor that promotes B cell conversion into Bregs and thus induces an immune-tolerance state (15). CTLA-4 is a glycoprotein belonging to the CD28

family which mediates the immune inhibitory functions of MSCs by suppressing immune-active T and B cells and induces the differentiation of these cells toward inhibitory phenotypes (50). Moreover, CTLA-4 induces the secretion of IDO from MSCs. In addition, CTLA-4 expression potentiates the PI3K signalling pathway in MSCs (51, 52). IL-10 is a cytokine that has many immunosuppressive effects on B cells including promoting formation of a Bregs phenotype and promoting plasma cells secretion of IL-10 (53). Complement system activation is required to stimulate the migration and maturation of B cells. MSCs express CD59 which suppress activation of complement system and as a result eliminate complement system activation of B cells (54). Our findings implicate Taz in the regulation of the immunomodulatory functions of MSCs and indicate that Taz deficiency in MSCs may result in the adoption of a more immunosuppressive state.

MSCs are known to induce an immune tolerance state, abrogate activation of B cells and dictate B cell differentiation fate. MSCs inherent immunosuppressive abilities may diminish the proliferation and activation of B cells and switch their phenotype toward potent immune-suppressive cells including Bregs and IL-10 secreting plasma cells (55, 56). Excessive accumulation of plasma cells is associated with the development of many autoimmune diseases and allograft rejection, while under-stimulation of these cells produces weaken short-term immunity (13, 57). We observed that the plasma cells induced by co-culture of B cells with TazKD MSCs were a specialized subset known as IL-10 secreting plasma cells which exhibit a powerful anti-inflammatory ability and produce an immune tolerance state that may subdue the immune response. In addition, the percentage of undifferentiated plasmablasts was higher in B cells co-cultured with TazKD MSCs. Furthermore, the ratio of plasmablasts/plasma cells was elevated in B cells co-cultured with TazKD MSCs indicating that TazKD MSCs reduced the differentiation potential of B cells into antibody-secreting plasma cells that intensify the immune reaction. IL-10 secreting Bregs play a central role in maintaining immunological

homeostasis by skewing T cells differentiation toward potent immunosuppressive T regulatory cells and inhibit many pro-inflammatory immune cells (58). The percentage of IL-10 secreting Bregs, IL-10 producing plasma cells and Sca-1^{+}/IgD immature B cells were increased by co-culture of TazKD MSCs with B cells. Thus, TazKD MSCs induced a shift in B cell differentiation towards a more highly immunosuppressive B cell phenotype.

A limitation of our study is that there is still significant residual Taz protein present in TazKD MSCs. How this would potentially affect the phenotype and relevance to Barth syndrome as a disease is currently unknown. Interestingly, bone marrow derived MSCs suppress production of ROS and extracellular traps in activated neutrophils, suggesting that MSCs can potentially limit the intensity of a respiratory burst upon inflammatory stimulation (59–62). Thus, it is possible that an enhanced immunosuppressive function of Taz-deficient MSCs could impact neutrophil function in Barth Syndrome patients and contribute to an increased susceptibility to infection.

In conclusion, our study has demonstrated for the first time that Taz deficiency in MSCs result in modulation of their immunosuppressive activity through increased expression of immunosuppressive markers. In addition, co-culture of Taz deficient MSCs with WT B cells significantly inhibits B cell proliferation, decreases B cell IgM secretion, increases B cell IL-10 production and alters B cell phenotype by inducing formation of immunosuppressive IL-10 secreting plasma cells and IL-10 secreting Bregs. A summary of our findings is presented in Fig. 6.

Figure 6

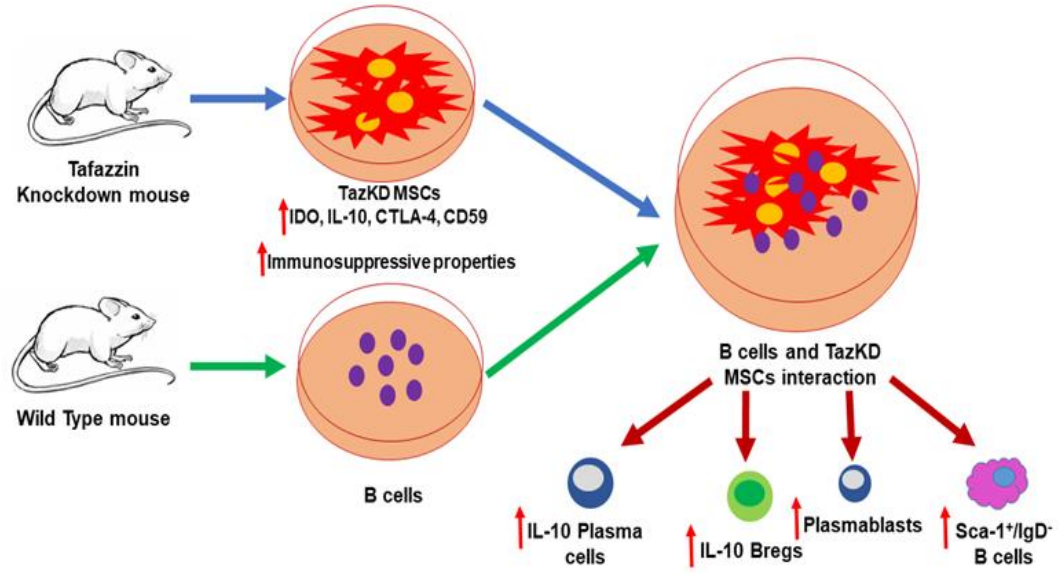


Figure 6. Taz deficiency in MSCs promote reprogramming of activated B lymphocytes toward potent immunosuppressive phenotypes. Mesenchymal stem cells (MSCs) from tafazzin knockdown (TazKD) mice exhibit an increase in immunosuppressive markers. When co-incubated with activated wild type B cells, MSCs from TazKD mice induce B cell differentiation into IL-10 secreting plasma cells and IL-10 secreting Bregs, undifferentiated plasmablasts and immature Sca-1^{+}/IgD B cells.

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Author contributions

H.M.Z., E.A.-E.-R., and G.M.H. were responsible for conceptual design of the study. H.M.Z, E.A.-E.R., G.C.S. performed the experiments and analyzed the data. F.O.-A. and A.J.M. designed, performed and analyzed the Flow cytometry experiments. L.K.C. was responsible of establishing and maintaining the Taz knockdown transgenic mouse model and culling the animals. H.M.Z., E.A.-E.-R., and G.M.H. interpreted the data, carried out statistical analysis and wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest statement

The authors of this study have no conflict of interest.

Data availability statement

The data that support the findings of this study are available in the methods and/or supplementary material of this article.

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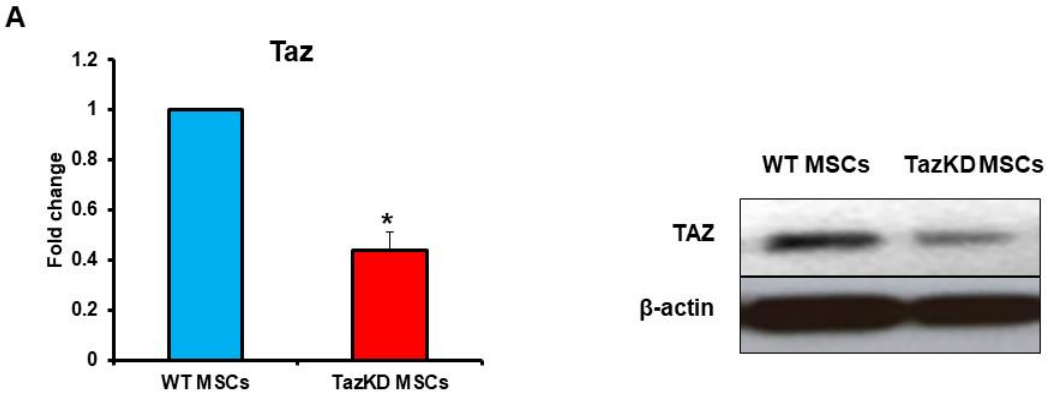
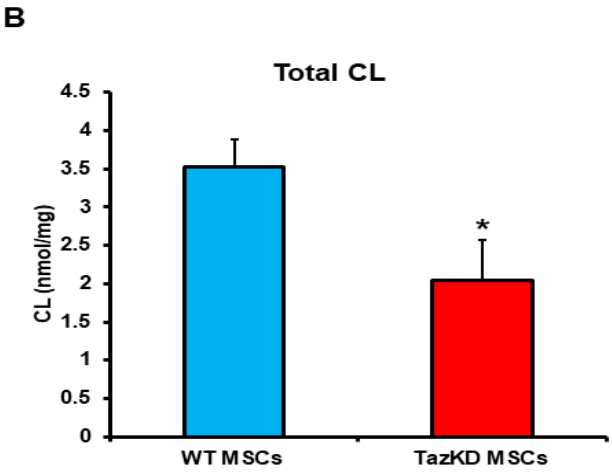
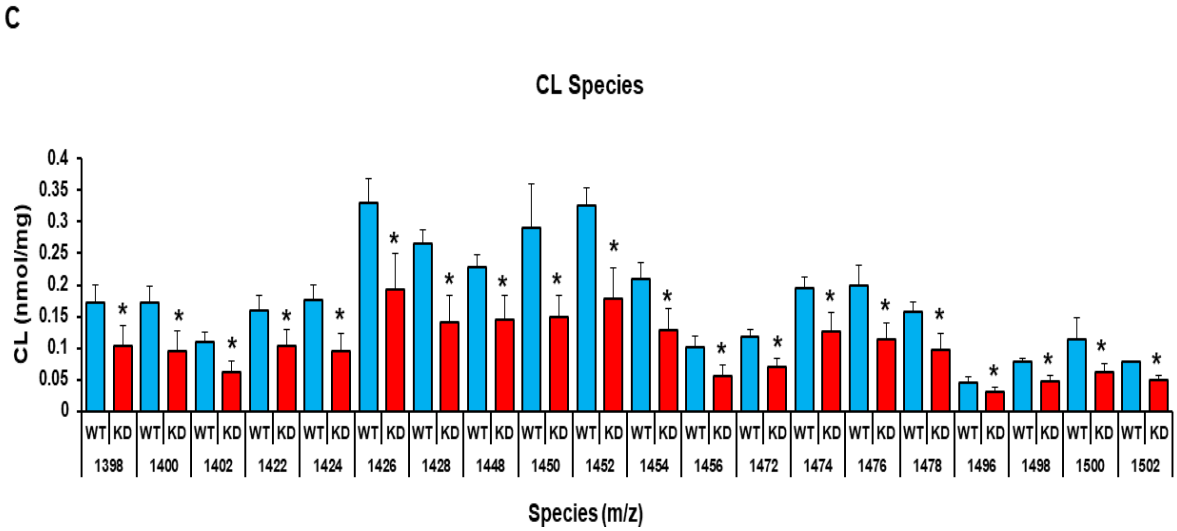
Supplementary Figure 1

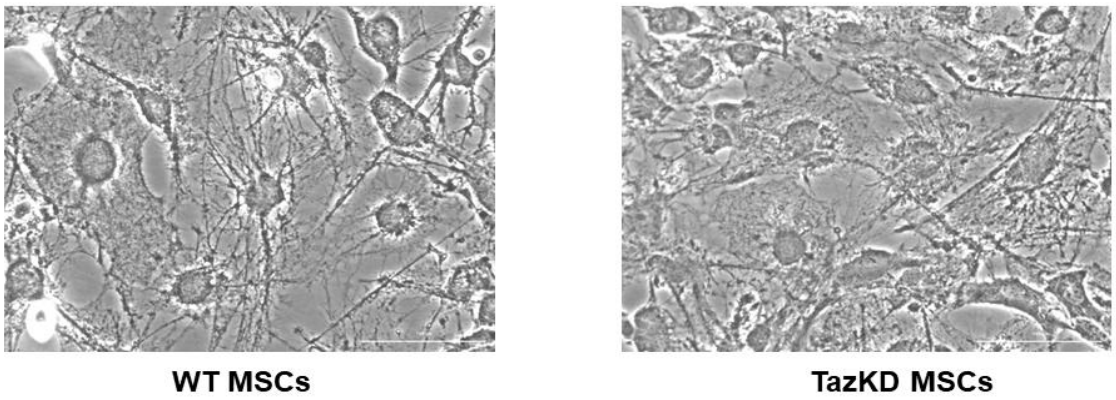






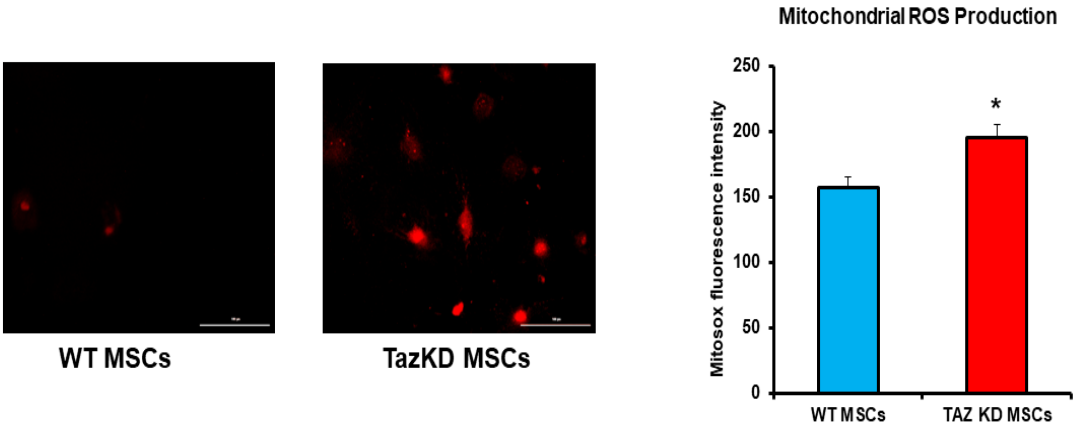
Supplementary Figure 1. Tafazzin deficiency reduces total CL and fatty acyl molecular species in TazKD MSCs. A. Taz protein expression in WT and TazKD MSCs. **B.** Total CL in WT and TazKD MSCs. **C.** Fatty acyl species CL in WT and TazKD MSCs. n=3. *p<0.05 compared with WT MSCs cells.

Supplementary Figure 2



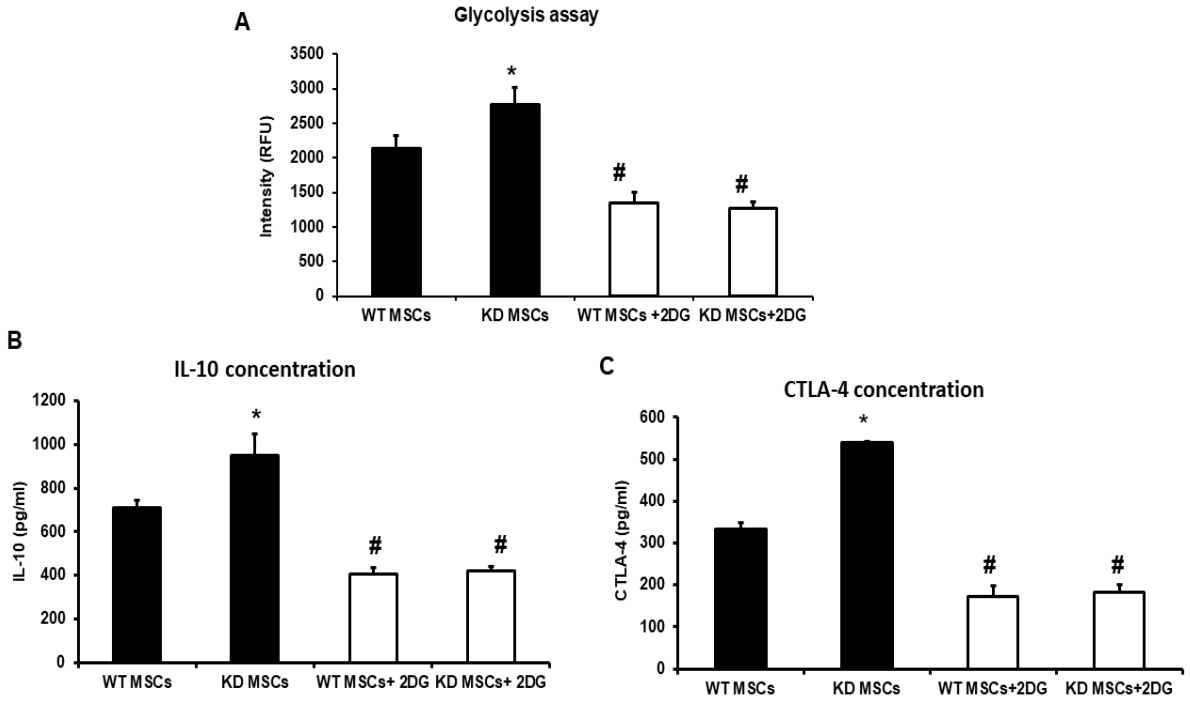
Supplementary Figure 2. Morphology of MSCs. Phase contrast images of WT MSCs and TazKD MSCs were taken by Cytation 5 imaging reader at 100 magnification scale.

Supplementary Figure 3



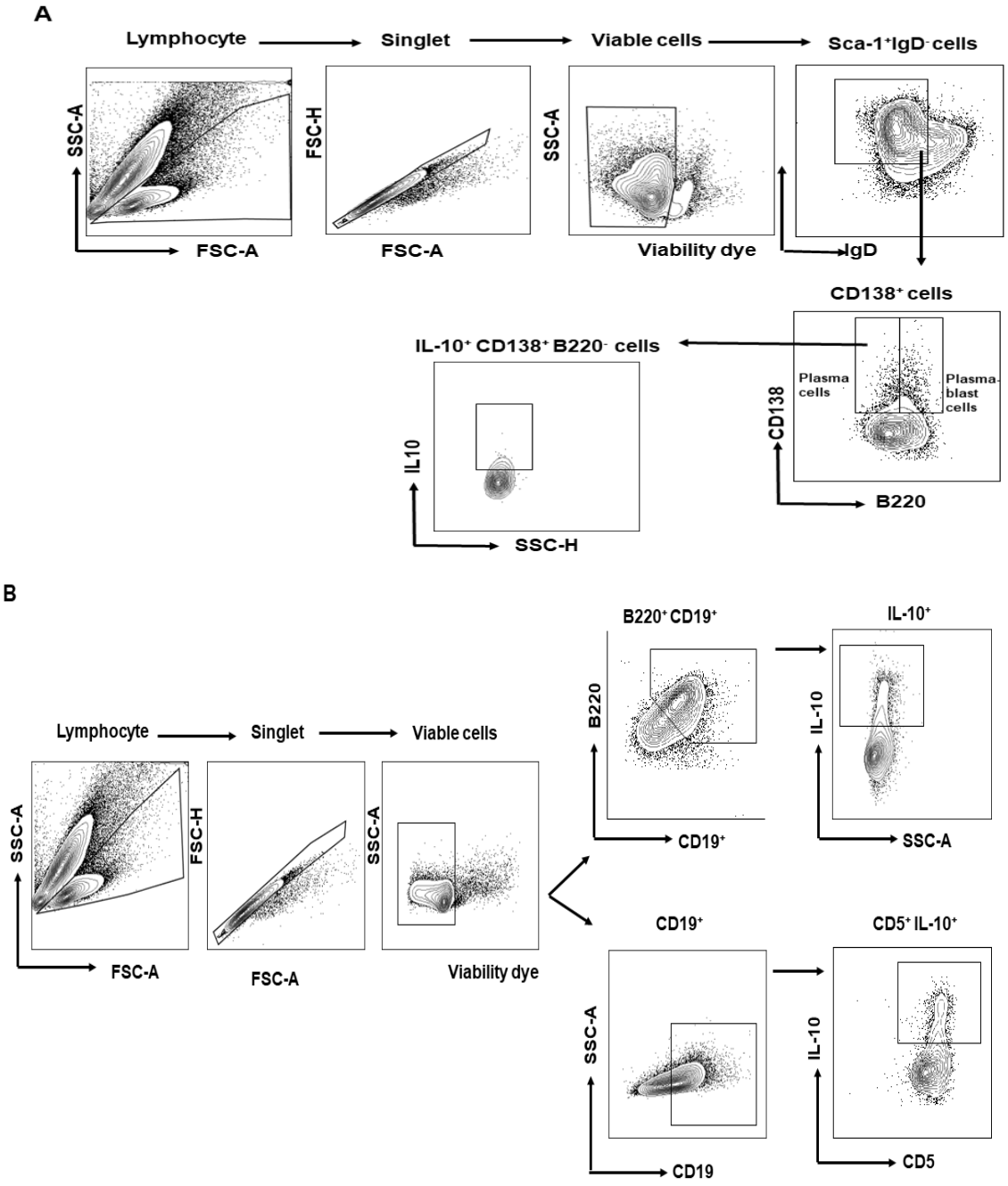
Supplementary Figure 3. Tafazzin deficiency enhances mitochondrial ROS production. Mitochondrial ROS levels were measured in WT and TazKD MSCs by using MitoSOX staining as described in Materials and Methods. n=3. *$p<0.05$ compared with WT MSCs cells.

Supplementary Figure 4



Supplementary Figure 4. Blocking glycolysis attenuated the elevated expression of immunosuppressive markers in TazKD MSCs. Glycolysis levels, IL-10 and CTLA-4 concentrations were measured as described in Materials and Methods. A. Glycolysis assay. B. IL-10 concentration. C. CTLA-4 concentration. n=4. *p<0.05 compared with WT MSCs cells. # p<0.05 compared with uninhibited cells.

Supplementary Figure 5



Supplementary Figure 5. Flow cytometry gating strategy for B cells subsets. A. Gating strategy for plasmablast cells, plasma cells, and IL-10 secreting plasma cells. **B.** Gating strategy for IL-10 secreting B cells and IL-10 secreting Bregs.

CHAPTER VII. Conclusions, Study Limitations and Future Directions

7.1 Conclusions:

BTHS is caused by mutation in the TAFAZZIN gene and is now considered a multisystem metabolic disorder [1]. The Taz protein controls the amount and molecular acyl species composition of CL [2]. Mature CL is essential for mitochondrial respiratory chain complexes to maintain energy generation via OXPHOS [3, 4]. Infection is the second leading cause of death in BTHS patients after cardiomyopathy. In addition, BTHS patients exhibit recurrent infections and they are more susceptible to develop serious complications [5]. We performed this study to better understand why BTHS patients develop immunodeficiency and serious infection. The effect of Taz deficiency on the immunological function of BTHS B lymphocytes had never been investigated. Many studies have used Epstein-Barr virus transformed lymphoblasts from BTHS patients to better understand the molecular mechanisms behind the disease [6–8]. However, these transformed B cells do not respond to stimulation the same way as naïve B cells. We thus examined the effect of Taz deficiency on the immunological properties of isolated murine B lymphocytes upon stimulation. Further, we studied how Taz deficiency may modulate the immunosuppressive function of bone marrow derived murine MSCs and investigated the impact that these Taz deficient MSCs have on B lymphocyte activity.

B cells play a key role in the adaptive immune response which are quickly respond to stimulation by increasing expression of their surface markers [9]. Our *in vitro* work showed that both Epstein-Barr virus transformed control and BTHS lymphoblasts do not increase surface marker expression after LPS stimulation. However, they express variable surface markers after CpG DNA activation. Epstein-Barr virus transformed B lymphoblasts are reprogrammed cells which exhibit different characteristics than naïve non-transformed B

lymphocytes. Therefore, we used *ex vivo* TazKD naïve B lymphocytes isolated from WT and TazKD mice to study the impact of Taz deficiency on B cell immunological function.

Naïve B lymphocytes remained in a quiescence state characterized by low metabolic activity and proliferation rate. However, LPS or anti-CD40 + IL-4 stimulated naïve B cells exhibited significantly increased metabolic and proliferative activities [10]. Our studies demonstrated for the first time that Taz deficiency negatively impacts the immunological characteristics of stimulated B cells and reduces their survival and proliferation by disturbing the mitochondrial respiratory chain complexes, particularly Complex IV. Mature CL is required for proper mitochondrial function [11]. Our results also demonstrated that Taz deficient B lymphocytes exhibit a marked decrease in total CL and its acyl species composition compared to WT B lymphocytes which is responsible for the diminished mitochondrial OXPHOS. Interestingly, our results also demonstrated that glycolysis, glycolytic capacity, and glycolytic reserve were reduced in the Taz deficient B cells. These results correlated with lower CL levels. Our results indicated that Taz deficiency disturbed major metabolic pathways in B cells leading to a significant decline in their activation, proliferation, and differentiation. We found that Taz deficiency reduced the percentage of B cells in the S/G2 phase and increased the percentage of B cells in the G0/G1 phase of the cell cycle. The PI3K pathway is required to support B cell proliferation. Our data demonstrated that PI3K, pAKt, and pAKt/AKt protein levels were significantly reduced in Taz deficient B cells. In addition, we demonstrated that Taz deficiency in activated B lymphocytes results an inhibition of their ability to induce surface marker expression including CD86 and CD69, decreased IgG and IgM synthesis and production, and reduced secretion of chemokines such as KC and MIP-2. These results correlated with the metabolic and proliferative activity impairment. Taz deficiency in B cells reduced 26S proteasome and immunoproteasome activities which are essential for proper immune function. Moreover, our proteomic analysis revealed alterations in the level of several

proteins that regulate mitochondrial function, survival, immunogenicity, and proteasomal function. Taz deficiency not only inhibited the T-independent pathway of B cell activation, but additionally inhibited the T-dependent pathway which may impede the ability of T cells to activate B cells through CD40L interaction.

We uncovered a unique association between Taz deficiency and the immunosuppressive properties of MSCs. Taz deficiency potentiated the immunomodulatory function of MSCs which in turn caused a shift in the phenotype of LPS activated B cells toward immunosuppressive phenotypes. An elevated glycolysis rate in MSCs is known to increase their ability to secrete immunosuppressive markers such as IDO [12]. Taz deficiency promoted the formation of immunosuppressive MSCs through enhancing glycolysis without impacting on mitochondrial OXPHOS. The increased immunosuppressive properties of Taz deficient MSCs may be considered as another possible mechanism for the reduced immune function of activated B cells since Taz deficiency in MSCs induced the differentiation of B cells to IL-10 secreting B cells and Bregs which strongly inhibit the immune response. Recently, B cell lymphopenia and hypogammaglobulinaemia was identified in a BTHS patient [13]. Thus, our findings may help in better understanding the possible mechanisms of why BTHS patients exhibit increased infections and may provide new insights into the design of targeted therapy that can promote the survival and improve the quality of life for BTHS patients. Therefore, targeting B cells would be a potential therapeutic approach to decrease infectious diseases in BTHS patients. Monitoring B cell counts, measuring serum immunoglobulins and antibodies function could help to treat recurrent infections in BTHS patients through use of prophylactic antibiotics and intravenous immunoglobulin replacement therapy.

7.2 Study limitations:

The transformed human lymphoblasts we used are considered a limitation of our model as our *in vitro* cellular work showed that Epstein-Barr virus transformed control and BTHS

lymphoblasts responded differently to activation through variability in their surface markers expression. Further experiments should be done to validate the current results using primary control and BTHS lymphoblasts. Our study is the first to examine the role of tafazzin in B lymphocyte immune function. We used the doxycycline-inducible TazKD mouse model to investigate the function of B lymphocytes. Despite the fact we used appropriate controls, the off target effects of doxycycline is considered a limitation for this model. Our *ex vivo* studies reported novel findings in the context of the immunological function of tafazzin deficient B lymphocytes and MSCs. Therefore, studying the effect of tafazzin deficiency in other immune cells would help to better understand immune function in BTHS patients. Another limitation in our studies was that significant residual Taz protein is present in TazKD B lymphocytes and MSCs. Therefore, further studies can be performed by knocking out tafazzin in WT murine B cells and MSCs or primary human B cells and MSCs to validate our findings.

7.3 Future Directions:

The findings of our studies will help in better understanding of the various mechanisms behind the immunodeficiency in BTHS patients. It is already known that BTHS patients suffer from recurrent and serious infections due to neutropenia. However, the impact of Taz mutations on the activity of other immune cells and its relation with the immunodeficiency in BTHS patients is not fully understood and is an area that should be investigated. The outcomes of our studies should be confirmed by studies on naïve B lymphocytes from BTHS patients. The reported changes in the major metabolic pathways in B cells should be examined *in vivo* in Taz deficient mice after triggering the immune response via injection with antigens such as LPS. This will aid in evaluating the *in vivo* function of B cells and the impact of other immune cells on B cell properties. One of the major complications in BTHS patients is cardiomyopathy leading to heart failure. The impact of the immune system on cardiac phenotypes in BTHS patients is still unknown. Therefore, future studies can be directed at examining if there is any

possible correlation between immunodeficiency and cardiomyopathy in BTHS patients through studying the effect of altered immune function of B cells and its contribution to the onset of cardiac complications. Furthermore, our findings can be extrapolated and tested in other immune cells to expand our understanding of the poor immunity in BTHS patients. Since our results have revealed that Taz deficiency modulates the immunosuppressive of MSCs, future studies should focus on exploring the mechanistic role of the Taz protein in triggering the release of high amounts of various immunosuppressive cytokines and factors by investigating the link between Taz and the upstream and downstream targets of the signalling pathways that control expression of each of these markers. Assessment of the role of Taz in immune cell function may help in developing new therapeutic targets for BTHS patients.

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