ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

INVESTIGATION OF THE MITOCHONDRIAL METABOLISM OF *HELICOBACTER*-ACTIVATED B CELLS

M.Sc. THESIS

Zeynep Nur ŞENTÜRK

Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

DECEMBER 2022

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

INVESTIGATION OF THE MITOCHONDRIAL METABOLISM OF *HELICOBACTER*-ACTIVATED B CELLS

M.Sc. THESIS

Zeynep Nur ŞENTÜRK
(521191125)

Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

Thesis Advisor: Prof. Dr. Ayça SAYI YAZGAN

DECEMBER 2022

ISTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

HELİKOBAKTER İLE AKTİVE EDİLMİŞ B HÜCRELERİNİN MİTOKONDRİYAL METABOLİZMASININ ARAŞTIRILMASI

YÜKSEK LİSANS TEZİ

Zeynep Nur ŞENTÜRK
(521191125)

Moleküler Biyoloji-Genetik ve Biyoteknoloji Anabilim Dalı

Moleküler Biyoloji-Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Prof. Dr. Ayça SAYI YAZGAN

ARALIK 2022

Zeynep Nur ŞENTÜRK, a M.Sc student of ITU Graduate School student ID 521191125, successfully defended the thesis/dissertation entitled "INVESTIGATION OF THE MITOCHONDRIAL METABOLISM OF *HELICOBACTER*-ACTIVATED B CELLS", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor: **Prof. Dr. Ayça SAYI YAZGAN** .....
Istanbul Technical University

Jury Members: **Prof. Dr. Ayten YAZGAN KARATAŞ** .....
Istanbul Technical University

Assoc. Prof. Devrim ÖZ ARSLAN .....
Acıbadem University

Date of Submission : 25 November 2022
Date of Defense : 07 December 2022

To my beautiful family and my best friends,

FOREWORD

First of all, I would like to thank my advisor Prof. Dr. Ayça SAYI YAZGAN for giving me this opportunity to carry out my graduation project in her Molecular Immunology Laboratory and also for her support and guidance.

I would like to thank my thesis committee members Prof. Dr. Ayten YAZGAN KARATAŞ And Assoc. Prof. Dr. Devrim ÖZ ARSLAN for devoting their precious time to my presentation.

Also, I want to thank my lab mates Mehmet ÇALÇI, Bahar DENİZ, Fatma Öykü ELASLAN, Işılay AKDAĞ, Niran ÖZDEMİR, and Zeynep Ece YILDIZ who become my little family at MOBGAM. Your patience, your friendship, and your laughter will be with me all the time. You make MOBGAM another home to me, thank you again for being together for 3.5 years.

As immunology groups, we become a huge family and Ece Oylumlu Altın and Göksu Üzel TURAN are the main parts of it. I am grateful for meeting you and thanks for your beautiful friendship.

I would like to thank TUBITAK (Project no: 119S447) and the Research Fund of Istanbul Technical University (ITU-BAP, Project no: 42944) for the financial support for both our 1001 project and my master thesis project.

I also need to thank my best friends Mısra GÖZ and Fatma Tüz Zehra KIZIL for their endless support and our amazing friendship. Also, all of my high school friends, my other family and Fiçuw members, I am grateful for being and growing with you. I can't imagine a life without all of you.

And I owe a special thanks to my beloved family; my father Hamit ŞENTÜRK and my mother Semiha ŞENTÜRK. I am so lucky to have you by my side and be your daughter. Thanks for your support, our endless tea and coffee parties, and your existence.

December 2022 Zeynep Nur ŞENTÜRK

TABLE OF CONTENTS

Page

ABBREVIATIONS

µg	**:**Microgram
µl	**:**Microliter
µM	**:**Micromolar
2-ME	**:**Beta mercaptoethanol
B2M	**:**Beta 2-microglobulin
BCA	**:**Bicinchoninic acid
BCR	**:**B cell receptor
Breg	**:**Regulatory B cell
BSA	**:**Bovine Serum Albumin
CagA	**:**Cytotoxin-associated gene A
COX1	**:**Cytochrome c oxidase subunit
DAMP	**:**Danger-associated molecular pattern
DC	**:**Dendritic cell
DMSO	**:**Dimethyl Sulfoxide
DNA	**:**Deoxyribonucleic Acid
dNTP	**:**Deoxyribonucleotide
EAE	**:**Experimental autoimmune encephalitis
EDTA	**:**Ethylenediaminetetraacetic acid
ELISA	**:**Enzyme-linked immunosorbent assay
ETC	**:**Electron transport chain
FBS	**:**Fetal bovine serum
FCCP	**:**Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
g	**:**Gram
GFP	**:**Green fluorescent protein
GLUT1	**:**Glucose transporter 1
h	**:**Hour
H. felis	**:***Helicobacter felis*
H. pylori	**:***Helicobacter pylori*
HRP	**:**Horseradish Peroxidase
HSP	**:**Heavy light strand
IFN	**:**Interferon
IMM	**:**Inner mitochondrial membrane
LPS	**:**Lipopolysaccharide
LRR	**:**Leucine rich-repeats
LSP	**:**Light strand promoter
LTA	**:**Lipoteichoic acid
MFI	**:**Mean fluorescence intensity
mg	**:**Milligram
min	**:**Minute
ml	**:**Milliliter
mM	**:**Millimolar
mtDNA	**:**Mitochondrial DNA
mtROS	**:**Mitochondrial reactive oxygen species

MZ :Marginal Zone
nDNA :Nuclear DNA
NKT :Natural Killer T cells
nm :Nanometer
nM :Nanomolar
OAA :Oxaloacetic acid
OMM :Outer mitochondrial membrane
OXPHOS :Oxidative phosphorylation
PAMP :Pathogen-associated molecular pattern
PBS :Phosphate buffered saline
PD-L1 :Programmed death-ligand 1
PE :Phycoerythrin
pg :Picogram
POLRMT :Mitochondrial RNA polymerase
Q-PCR :Quantitative Polymerase Chain Reactions
RNA :Ribonucleic Acid
ROS :Reactive oxygen species
rpm :Round per minute
RPMI :Roswell Park Memorial Institute Medium
RPS18 :18S ribosomal subunit gene
RT :Room temperature
RT-PCR :Real Time-Polymerase Chain Reaction
SDH :Succinate dehydrogenase
T2-MZP :Transitional 2 marginal-zone precursor cells
TCA :Tricarboxylic acid cycle
TD :Thymus-dependent
Tfam :Mitochondrial transcription factor A
Tfb1m :Mitochondrial transcription factor B1
Tfb2m :Mitochondrial transcription factor B2
TI :Thymus-independent
TIR :Toll/IL-1 receptor
TLR :Toll-like receptor
VacA :Vacuolating cytotoxin A
ΔΨm :Mitochondrial membrane potential

LIST OF TABLES

Page

LIST OF FIGURES

Page

INVESTIGATION OF THE MITOCHONDRIAL METABOLISM OF *HELICOBACTER*-ACTIVATED B CELLS

SUMMARY

Helicobacter pylori (*H. pylori*) is a gram-negative, microaerophilic, and spiral-shaped bacterium and a member of the *Helicobacteraceae* family. *H. pylori* was discovered in 1982 by Warren and Marshall. *Helicobacter* infection can lead to multiple gastro pathologies such as chronic gastritis, gastric cancer, peptic ulcer disease, and mucosa-associated lymphoid tissue lymphoma. Whereas more than 50% of the world population has been infected with *H. pylori*, 80% of them are asymptomatic. Similar to *H. pylori*; *H. felis* is a gram-negative, urease-positive, spiral-shaped, and microaerophilic bacteria. Studies have shown that *H. felis* can induce gastric atrophy, metaplasia, dysplasia, chronic and persistent inflammatory response, and gastric cancer in mice models. Because *H. pylori* have less capacity to activate an efficient immune response in mice compared to *Helicobacter felis* (*H. felis*); *H. felis* is used to generate mice models for studying this pathogen.

B cells play critical roles in adaptive immunity with antigen presentation to T cells and antibody production. Recently, new B cell subsets have been shown to exert anti-inflammatory and immune suppressive properties were discovered. These B cells are termed regulatory B cells (Bregs) by Bhan and colleagues. In mice; mainly $CD19^{+}CD21^{hi}CD23^{hi}CD24^{hi}$ transitional 2 marginal-zone precursor cells (T2-MZP), IL-10 producing $CD1d^{hi}CD5^{+}$ B10 cells, $CD19^{+}CD21^{hi}$ $CD23^{-}$ marginal-zone (MZ) B cells, Tim-1^{+} B cells, $CD19^{+}CD5^{+}$ B1a cells, $CD9^{+}$ B cells, $CD138^{+}$ plasma B cells, and $CD138^{+}CD44^{hi}$ plasma blasts are identified as Breg subsets. For maintaining host immune tolerance and balance effector immune responses, these Breg cells secrete IL-10, IL-35, and TGF-β cytokines. In addition to cytokines, Breg cells also use cell membrane-bound molecules such as CD39, CD73, programmed death-ligand 1(PD-L1), or aryl hydrocarbon receptors for their functions. Stimulation of B cells with *H. felis*; signals via TLR2 and MyD88 and results with IL-10 producing Bregs. Bregs can induce differentiation of naive $CD4^{+}$ T cells to IL-10-producing regulatory Tr1 cells by direct B and T cell interactions for suppressing *Helicobacter*-associated pathologies.

Metabolism is the collection of all anabolic and catabolic reactions which are the generation and breakdown of cellular substances respectively. Oxidative phosphorylation (OXPHOS) is one of the major metabolic pathways inside the cell. It occurs in the mitochondria and consists of the tricarboxylic acid cycle (TCA) and electron transport chain reactions for generating ATP. Shortly, in OXPHOS electrons formed from the tricarboxylic acid cycle (TCA); are combined with molecular oxygen (final acceptor of electron transport chain) and this results in many oxidation/reduction reactions where energy is released for the production of ATP from ADP. Mitochondria are double membrane organelles that have both outer and inner mitochondrial membranes (OMM and IMM) and are found in eukaryotic cells. Membrane

transporters and electron transport chain (ETC) complexes of mitochondria localize on the inner mitochondrial membrane. IMM encloses a viscous structure called a mitochondrial matrix. Enzymes, mitochondrial DNA (mtDNA), ribosomes, and nucleotides are placed in the mitochondrial matrix. Mitochondrial DNA encodes 37 mitochondrial genes including 22 transfer RNAs, 2 ribosomal RNAs, and 13 important oxidative phosphorylation polypeptides: ND1, ND2, ND3, ND4L, ND4, ND5, ND6 (parts of Complex I); Cytochrome b (parts of Complex III), COI, COII, COIII (parts of Complex IV) and ATP6, ATP8 (parts of Complex V). To provide expression of a mitochondrial gene, mitochondrial DNA needs to be transcribed by mitochondrial transcription factor A (Tfam), mitochondrial RNA polymerase (POLRMT), and mitochondrial transcription factor B1 and B2 (Tfb1m and Tfb2m).

Two of the most critical features of multicellular life are metabolism and immunity. These can be explained as the need to distribute nutrients across cells, tissues & organs and protect from injury and inflammation. In recent years, studies have focused on elucidating the metabolism of immune cells in the context of their survival, activation, differentiation, and functions. For the activation of immune cells, signals which are triggered by metabolic intermediates and ATP molecules are required. In order to maintain proper immune cell activation, differentiation, and function, mitochondrial metabolism which generates energy plays a critical role. Studies have demonstrated that B cell activation with B cell receptor (BCR) or different Toll-like receptor (TLR) ligands changes mitochondrial dynamics. LPS-stimulated B cells enhance mitochondrial mass, and co-stimulation of B cells with BCR ligand IgM and TLR9 ligand CpG increases mitochondrial biogenesis. In addition to that, anti-CD-40 and IL-4 stimulated B cells to undergo OXPHOS. However, there is no information in the literature about the mitochondrial metabolism of *Helicobacter*-activated B cells.

The main aim of this study is to elucidate the mitochondrial metabolism of *Helicobacter*-infected B cells. For this purpose, B cells were magnetically isolated from spleens of C57BL6 mice and treated with *H. felis* antigen, PAM3CSK4, and LPS for 6h, 24h, and 48h. Afterwards, cells were collected at respective time points for mitochondrial mass and membrane potential staining by using Mitoview Green and Mitoview 633 or TMRE dyes respectively in the flow cytometry. The supernatant of these cells is used for the IL-10 ELISA experiments for checking their IL-10 secretion.

H. felis, PAM3CSK4, and LPS-stimulated B cells increased their IL-10 production most noticeably at 24h and 48h indicating the suppressive capacity of that cells. Also, compared to the unstimulated control group, all of the stimulant groups increased both the mass and membrane potential of mitochondria at 24h and 48h time points.

The second aim of our study was to investigate whether B cells with high mitochondrial membrane potential (Mitoview 633^+ B cells) produce IL-10 or not. For that, after B cells were isolated from IL-10 GFP reporter (VertX IL10 ^{egfp}) mice, they were treated with *H. felis* antigen, PAM3CSK4, and LPS for 6h, 24h, and 48h. Mitochondrial membrane potential were analyzed by flow cytometry. Afterwards, at 6h, 24h and 48h we evaluated mitochondrial membrane potential of the IL-10 producing B cells with quadrant analysis using flow cytometry. At 6h time there were no significant changes on IL-10^+ Mitoview 633^+ B cells. But at 24h and 48h time points; *H.felis*, PAM3CKS4, and LPS-stimulated B cells increased IL-10^+ Mitoview 633^+ B cells. These data show that in all stimulated B cell groups; high portion of the IL-10 producing B cells also have high mitochondrial potential. Our data shows, *H. felis*, PAM3CSK4, and LPS-stimulated Mitoview 633^+ B cells increased their IL-10

GFP signal both at 24h and 48h time points compared to the unstimulated control group.

The third aim of our study was to investigate mitochondrial biogenesis markers: mtDNA:nDNA ratio and mitochondrial transcription factor A (Tfam) gene expression. To perform Q-PCR experiments isolated and treated B cells were collected for DNA and RNA isolation at 6h, 24h, and 48h. For mtDNA:nDNA ratio analysis in Q-PCR; respectively cytochrome c oxidase subunit I (COX1) and 18S ribosomal subunit gene (RPS18) were targeted by using gene-specific primers. While *H. felis*, PAM3CSK4, and LPS-stimulated B cells decreased their mtDNA:nDNA ratio, they increased Tfam expression level compared to the unstimulated control group.

This study showed that *H. felis*-activated B cells have active/functional mitochondria, and increased oxidative phosphorylation for energy production and their IL-10 production can be related to their mitochondrial metabolism.

HELİKOBAKTER İLE AKTİVE EDİLMİŞ B HÜCRELERİNİN MİTOKONDRİYAL METABOLİZMASININ ARAŞTIRILMASI

ÖZET

Helikobakter pilori (*H.pilori*) gram-negatif, mikroaerofilik ve spiral şekilli bir bakteri olup *Helicobacteraceae* ailesine aittir. *H.pilori* 1982 yılında Warren ve Marshall tarafından keşfedilmiştir. *Helikobakter* enfeksiyonu kronik gastrit, gastrik kanser, peptik ülser hastalığı ve mukoza ile ilişkili lenfoid doku lenfoması gibi birçok gastro patolojiye yol açabilir. Dünya nüfusunun %50'si *H. pilori* ile enfekte olup, *H. pilori* ile enfekte bireylerin %80'i asemptomaktir. *H. pilori*' ye benzer şekilde , *H.felis*'de gram negatif, üreaz-pozitif, spiral şekilli ve mikroaerofilik bir bakteridir. Fare modellerinde yapılan çalışmalar, *H.felis*'in gastrik atrofi, metaplazi, displazi, kronik ve kalıcı inflamasyon ve gastrik kansere sebep olduğunu göstermiştir. *H. pilori*'nin farelerde etkili bir immün cevap oluşturma kapasitesi düşük olduğu için, bu patojenin çalışılabilmesi amacıyla fare modelleri geliştirilmesinde *Helikobakter felis* (*H.felis*) kullanılmaktadır.

B hücreleri T hücrelerine antijen sunumu yaparak ve antikor üreterek adaptif immünitede önemli rol oynarlar. Son zamanlarda, anti inflamatuar ve immün sistemi baskılayıcı özellikleri olan yeni B hücre alt tipleri keşfedilmiştir. Bu B hücreleri Bhan ve çalışma arkadaşları tarafından regülatör B hücreler (Breg) olarak adlandırılmıştır. $CD19^{+}CD21^{hi}CD23^{hi}CD24^{hi}$ translasyonel 2 marjinal zon hücreleri (T2-MZP), IL-10 üreten $CD1d^{hi}CD5^{+}$ B10 hücreleri, $CD19^{+}CD21^{hi}$ $CD23^{-}$ marjinal zon (MZ) B hücreleri, Tim-1^{+} B hücreleri, $CD19^{+}CD5^{+}$ B1a hücreleri, $CD9^{+}$ B hücreleri, $CD138^{+}$ plazma B hücreleri ve $CD138^{+}CD44^{hi}$ plazmablast Breg alt grupları farelerde tanımlanmış regülatör B hücreleridir. Regülatör B hücreler immün tolerans ve efektör immün hücre yanıtını dengelemek için IL-10, IL-35 ve TGF-β sitokinlerini salgılar. Bu sitokin moleküllerine ek olarak, Breg hücreleri immün sistemi baskılayıcı fonksiyonlarını gerçekleştirmek için CD39, CD73, programlanmış ölüm ligandı 1 (PD-L1) ve aril hidrokarbon reseptörleri gibi hücre zarına bağlı diğer molekülleri de kullanmaktadırlar. *H. felis* B hücrelerini TLR2 ve Myd88 sinyal yolağı aracılığı ile indükler ve bu indükleme IL-10 üreten B hücreleri ile sonuçlanır. Breg hücreleri *Helikobakter* ilişkili patolojileri baskılayabilmek için T hücreleri ile etkileşime girerek naif $CD4^{+}$ T hücrelerinin IL-10 üreten regülatör T hücrelerine farklılaşmasını sağlar.

Metabolizma, sırasıyla hücrede yer alan moleküllerin yapım ve yıkımlarını içeren anabolik ve katabolik reaksiyonlardan oluşmaktadır. Oksidatif fosforilasyon (OXPHOS) hücre içinde gerçekleşen en önemli metabolik yolaklardan biridir. Oksidatif fosforilasyon mitokondride gerçekleşir ve enerji üretiminde görev alan trikarboksilik asit ve elektron transport zinciri reaksiyonlarını içermektedir. Oksidatif fosforilasyonda kısaca, trikarboksilik asit döngüsü sonucunda elektron transport zincirine aktarılan elektronlar, oksijen molekülü ile birleşir ve oksidasyon/redüksiyon reaksiyonları sonucunda ADP molekülünden ATP oluşumu ile hücrelerden enerji salınımı gerçekleşir.

Mitokondri, ökaryotik hücrelerde bulunan ve hem dış hem de iç mitokondriyal membrana sahip çift membranlı bir organeldir. Membran transportları ve elektron transport zinciri mitokondriye ait iç mitokondriyal membranda (İMM) yer almaktadır. İMM içerisinde mitokondriyal matriks olarak adlandırılan viskoz bir yapı bulunmaktadır. Enzimler, mitokondriyal DNA (mtDNA), ribozomlar ve nükleotitler mitokondriyal matriks içinde yer almaktadır. Mitokondriyal DNA; 22 adet transfer RNA, 2 adet ribozomal RNA ve 13 adet oksidatif fosforilasyonda rol alan polipeptit (ND1, ND2, ND3, ND4L, ND4, ND5, ND6 (Kompleks I'in yapısına katılırlar); Cytochrome b (Kompleks III'ün yapısına katılırlar), COI, COII, COIII (Komplex IV'ün yapısına katılırlar) ve ATP6, ATP8 (Komplex V'in yapısına katılırlar)) olmak üzere 37 adet mitokondiyal geni kodlamaktadır. Mitokondriyal bir genin ekspresyonunun gerçekleşebilmesi için mitokondriyal DNA'nın mitokondriyal transkripsiyon faktörü A (Tfam), mitokondriyal RNA polimeraz (POLRMT) ve mitokondriyal transkripsiyon faktörü B1 ve B2 (Tfb1m ve Tfb2m) tarafından transkribe edilmesi gerekir.

Çok hücreli hayat için gerekli iki hücresel fonksiyon; sırasıyla besin maddelerinin hücre, doku ve organlar arasında dağıtılması ve organizmanın inflamasyondan korunması olarak tanımlanan metabolizma ve immünitedir. Son yıllarda çalışmalar; canlılık, aktivasyon, farklılaşma ve fonksiyonları kapsamında immün hücrelerin enerji metabolizmalarının aydınlatılmasına odaklanmıştır. İmmün hücrelerin aktivasyonunun sağlanabilmesi için gerekli sinyal yolakları; metabolik ara bileşenler ve ATP'ye ihtiyaç duyar. Bu yüzden enerji üretimini sağlayan mitokondriyal metabolizma; immün hücre aktivasyonu, farklılaşması ve fonksiyonunun uygun şekilde sürdürülmesinde kritik bir rol oynar. Çalışmalar B hücrelerinin, B hücre reseptörü (BCR) veya Toll-benzeri reseptörler (TLR) ile aktivasyonu sonucunda mitokondriyal dinamiklerini değiştirdiklerini göstermektedir. LPS ile uyarılan B hücreleri mitokondriyal kütlelerini artırırken, B hücre reseptör ligandı IgM ve TLR9 ligandı CpG ile eş zamanlı uyarılan B hücreleri ise mitokondriyal biogenezini artırmaktadır. Bunlara ek olarak, CD40 ve IL-4 ile stimule edilen B hücreleri oksidatif fosfororilasyon yapmaktadır. Ancak literatürde *Helikobakter* ile aktive edilen B hücrelerinin mitokondriyal metabolizmaları ile ilgili herhangi bir bilgi bulunmamaktadır.

Bu çalışmanın asıl amacı, *Helikobakter* ile aktive edilen B hücrelerinin mitokondriyal metabolizmasını aydınlatmaktır. Bu amaçla, öncelikle B hücreleri C57BL6 farelerinin dalaklarından manyetik olarak izole edildi ve 6, 24 ve 48 saat boyunca *H. felis* antijeni, PAM3CSK4, ve LPS ile uyarıldı. Daha sonrasında, hücreler belirtilen saat aralıklarında toplanarak, hücrelerin mitokondriyal kütle ve membran potansiyelleri sırasıyla Mitoview Green ve Mitoview 633 ya da TMRE boyaları kullanılarak akan hücre ölçerde analiz edildi. Bu hücrelerin süpernatantları, IL-10 salınım miktarlarının tayin edilebilmesi için IL-10 ELIZA deneyleri sırasında kullanıldı.

H. felis antijeni, PAM3CSK4, ve LPS ile stimüle edilen B hücreleri baskılayıcı kapasitelerinin göstergesi olarak IL-10 üretimlerini, 24 ve 48 saatte belirgin olarak artırmıştır. Ayrıca uyarılmamış kontrol grubuna kıyasla, uyarı alan tüm gruplar 24 ve 48 saatte hem mitokondriyal kütlelerini hem de mitokondriyal membran potansiyellerini artırmıştır.

Çalışmamızın ikinci amacı, yüksek membran potansiyeline sahip B hücrelerinin IL-10 sitokinini üretip üretmediğini göstermekti. Bu yüzden, B hücreleri IL-10 GFP reporter (VertX IL10 ^{egfp}) farelerin dalaklarından izole edildikten sonra, 6, 24 ve 48 saat

boyunca *H. felis* antijeni, PAM3CSK4, ve LPS ile stimüle edildi. Mitokondriyal membran potansiyeli ve Mitoview 633 pozitif B hücrelerinin IL-10 GFP sinyali akan hücre ölçer kullanılarak analiz edildi. Bu B hücreleri ayrıca Mitoview 633 ve IL-10 GFP sinyalleri bakımından "quadran" analizi ile değerlendirildi. Quadran analizi Mitoview 633^- IL-10^- (iki belirte. Içinde negative B hücre popülasyonu), Mitoview 633^- IL-10^+(sadece IL-10 pozitif B hücreleri), Mitoview 633^+ IL-10^- (sadece Mitoview 633 pozitif B hücreleri) ve Mitoview 633^+ IL-10^+ (iki belirteç içinde pozitif B hücre popülasyonu) olmak üzere 4 ayrı popülasyon göstermektedir. 6 saatte IL-10+ Mitoview 633+ B hücrele yüzdelerinde kontrol grubuna kıyasla herhangi bir değişiklik gözükmez iken; uyarı alan B hücreleri 24 ve 48 saatte IL-10+ Mitoview 633+ B hücre yüzdelerini kontrol grubuna göre anlamlı olarak artmıştır. Elde ettiğimiz veriler, *H. felis* antijeni, PAM3CSK4, ve LPS ile uyarılan Mitoview 633 pozitif B hücrelerinin uyarı almayan gruba kıyasla 24 ve 48 saatte IL-10 GFP sinyallerini artırdığını göstermektedir. Ayrıca; IL-10 üreten B hücrelerinin neredeyse tamamının aynı zamanda yüksek mitokondriyal potansiyele sahip olduğu gözlenmiştir.

Çalışmamızın üçüncü amacı ise mitokondriyal biogenez belirteçlerini araştırmaktı: mtDNA:nDNA oranı ve mitokondriyal transkripsiyon faktör A (Tfam) geninin ifade seviyesi. Q-PZR deneylerini gerçekleştirmek adına izole edilen ve uyarılan B hücreleri 6, 24 ve 48 saat sonunda toplanarak hücre pelletlerinden DNA ve RNA izolasyonu yapıldı. mtDNA:nDNA oranını Q-PZR'da tayin edebilmek için sırasıyla sitokrom c oksidaz subunit 1 ve ribozomal protein 18S genleri gen spesifik primerler kullanılarak hedef alındı. *H. felis* antijeni, PAM3CSK4, ve LPS ile uyarılan B hücreleri uyarı almayan gruba kıyasla mtDNA:nDNA oranlarını azaltırken, Tfam ifade seviyelerini artırmıştır.

Sonuç olarak bu çalışma; *H.felis* ile aktive edilen B hücrelerinin, aktif/fonksiyonel mitokondrilere sahip olduğunu, oksidatif fosforilasyonu artırdığını ve IL-10 üretimlerinin mitokondriyal metabolizmaları ile ilişkili olabileceğini göstermiştir.

1. INTRODUCTION

1.1 *Helicobacter pylori* and *Helicobacter felis*

Helicobacter pylori (*H. pylori*) is a member of the *Helicobacteraceae* family which was discovered in 1982 by Warren and Marshall. *H. pylori* is a spiral-shaped, microaerophilic gram-negative bacterium. It can lead to several gastro pathologies such as peptic ulcer disease, chronic gastritis, gastric cancer, and mucosa-associated lymphoid tissue lymphoma. While more than 50% of the world population has been infected with *H. pylori*, most of the infected people do not show any symptoms of the above-mentioned gastro pathologies. These bacteria use different virulence factors and specialized proteins, for the progression of complications and adhesion to the mucosal epithelium, such as vacuolating cytotoxin A (VacA), cytotoxin-associated gene A (CagA), PicB, BabA, SabA, or OpiA. Also, *H. pylori* have urease activity, allowing bacteria to live in an acidic environment such as the stomach where pH values change from 4 to 6.5 (Mégraud et al., 2015; Guevara & Cogdill, 2020).

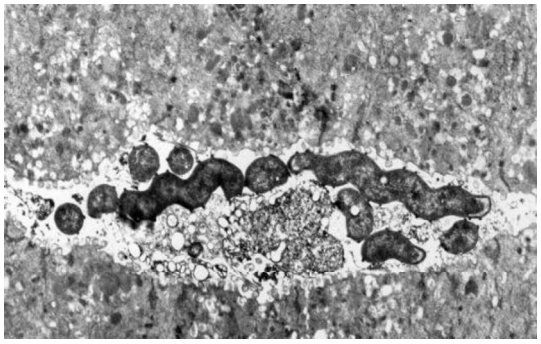


Figure 1.1: Electron micrograph of *H.felis* (Simpson et al., 2000).

Helicobacter felis (*H. felis*) is isolated from the stomachs of cats in 1990 by Lee et al. Bacteria are also found in the stomachs of dogs, rabbits, and cheetahs in addition to cats. Similar to *H. pylori*; *H.felis* is a gram-negative, urease-positive, spiral-shaped, and microaerophilic bacterium. Studies have shown that *H. felis* can induce gastric atrophy, metaplasia, dysplasia, chronic and persistent inflammatory response, and gastric cancer in mice models (Fox, 2002; Cai et al., 2005). These gastric pathologies display high similarity for *H. pylori-infected* individuals. Because *H. pylori* have less

capacity to activate an efficient immune response in mice compared to *H. felis*; *H. felis* is used to generate mice models for studying this pathogen.

1.2 Immune System and Its Components

The immune system is a collection of cells, structural and chemical barriers, and reactions that eliminate the "non-self" molecules to protect the host. These "non-self" molecules are called antigens and they can be bacteria, fungi, parasites, viruses, toxins, cancer cells, or particles that come from outside the host's body. When an antigen enters, the first line of defense being triggered is innate immunity. Innate immune responses are not antigen-specific and they occur immediately or within hours of coming across antigen molecules. In contrast to innate immunity, the second line of defense is antigen-specific and it is called adaptive immunity (Marshall et al., 2018). Adaptive immune responses take time due to antigen exposure for generating specific humoral (via B cells) or cellular immunity (via T cells).

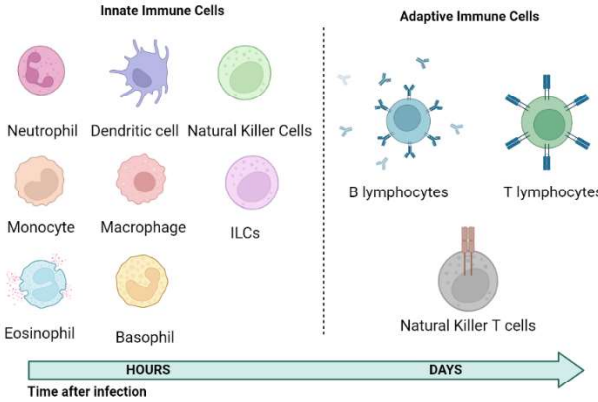


Figure 1.2: Cells of the innate and adaptive immune system (Adapted from Abbas et al, 8th edition; Illustrated in BioRender).

While monocytes, macrophages, dendritic cells (DCs), neutrophils, natural killer cells, basophils, mast cells, and eosinophils are the components of innate immunity; B, T, and natural killer T cells (NKT) are the cells of adaptive immunity. All of these immune cells use different mechanisms such as phagocytosis, activation of the complement system, production of cytokines, chemokines, or antibodies, and neutralization or opsonization of the pathogen with antibody molecules to eliminate foreign invaders (Abbas et al, 8th edition).

1.3 B Cells

B cells are one of the main cells of adaptive immunity and they originate in the bone marrow from hematopoietic stem cells. B lymphocytes produce antibody molecules to neutralize or eliminate pathogens by activation of the complement system or phagocytosis (Abbas et al, 8th edition). B cell development consists of early differentiation which occurs when antigens are absent; maturation; antigen recognition and antibody production. During development, B cells obtain two important features: the distinction between self-antigens from non-self-antigens and memory responses (Althuwaiqeb & Bordoni, 2021).

1.3.1 Activation of B cells

Activation of B cells is triggered by a specific B cell receptor (BCR) at first. BCRs can bind to antigens of pathogens and initiate a signaling cascade inside the cell. Also, they can present thymus-dependent (TD) antigens with MHC class II molecules to antigen-specific T helper cells. When antigenic peptides are recognized; T helper cells express cytokines and cell surface molecules or interact with CD40 of B cells via their CD40L to induce B cell survival, proliferation, and differentiation. Some of the B cell responses to microbial antigens do not require T cell help and are called thymus-independent (TI) antigens. TI antigens can induce the signaling of Toll-like receptors (TLRs) to produce antibodies (Murphy et al.,2017).

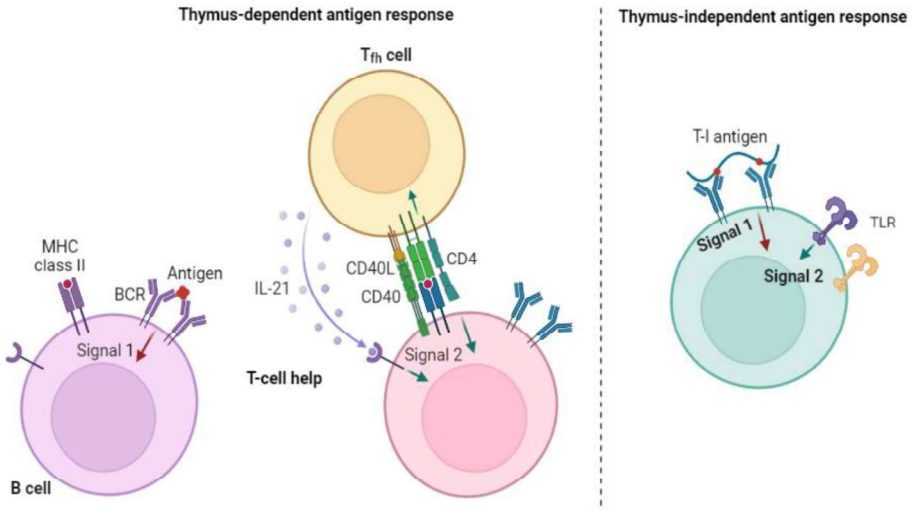


Figure 1.3: Activation of B cells by TD or TI antigens (Adapted from Abbas et al, 8th edition; Illustrated in BioRender).

1.3.2 Regulatory B cells

As mentioned above, B cells play a critical role in adaptive immunity with antibody production or antigen presentation to T cells. Additional functional roles and recent subsets of B cells were discovered with the help of autoimmune disease models of experimental autoimmune encephalitis (EAE), colitis, and arthritis. In the EAE model, B cell-deficient mice could not recover from excessive inflammation of the disease compared to wild-type mice (Wolf et al., 1996). Studies show that B cell suppression of inflammatory responses is provided by the secretion of IL-10 from these cells in EAE, colitis, and arthritis (Fillatreau et al., 2002; Mauri et al., 2003; Mizoguchi et al.,2002). B cell subsets that have been shown to exert these suppressive or anti-inflammatory properties for contributing to the maintenance of the host tolerance are termed " regulatory B cells (Bregs)" by Bhan and colleagues in 2002. The origin and development of Bregs are still unclear but many Breg subsets were discovered both in mice and humans. While in mice mainly $CD19^{+}CD21^{hi}CD23^{hi}CD24^{hi}$ transitional 2 marginal-zone precursor cells (T2-MZP), IL-10 producing $CD1d^{hi}CD5^{+}$ B10 cells, $CD19^{+}CD21^{hi}$ $CD23^{-}$ marginal-zone (MZ) B cells, Tim-$^{1+}$ B cells, $CD19^{+}CD5^{+}$ B1a cells, $CD9^{+}$ B cells ,$CD138^{+}$ plasma B cells and $CD138^{+}CD44^{hi}$ plasmablasts are identified as Breg subsets; in humans $CD19^{+}CD24^{hi}$ $CD38^{hi}CD1d^{hi}$ B cells, $CD19^{+}CD24^{hi}CD27^{+}$ B10 cells, $CD9^{+}$ B cells, $CD19^{+}CD25^{hi}CD71^{hi}$ $CD73^{-}$Br1 cells and $CD19^{+}CD24^{hi}CD27^{int}$ plasmablasts were described as Breg subsets (Rosser & Mauri, 2015; Jansen et al., 2021).

Activation, function, or differentiation of regulatory B cells are dependent on BCR and TLRs signaling, CD40-CD40L, and CD80/CD86-CD28 interactions (Mauri & Bosma, 2012).Stimulation of B cells with *H.felis*; signals via TLR2 and MyD88 and results with IL-10 producing Bregs. Bregs can induce differentiation of $CD4^{+}$ T cells to IL-10-producing regulatory Tr1 cells by direct B and T cell interactions for suppressing *Helicobacter*-associated pathologies (Sayi et al., 2011).

Cytokines such as IL-10, TGF-β, IL-35 or cell membrane-bound molecules such as CD39, CD73, programmed death-ligand 1(PD-L1) or aryl hydrocarbon receptors are examples of key molecules which are associated with Breg's suppressive functions (Jansen et al., 2021).

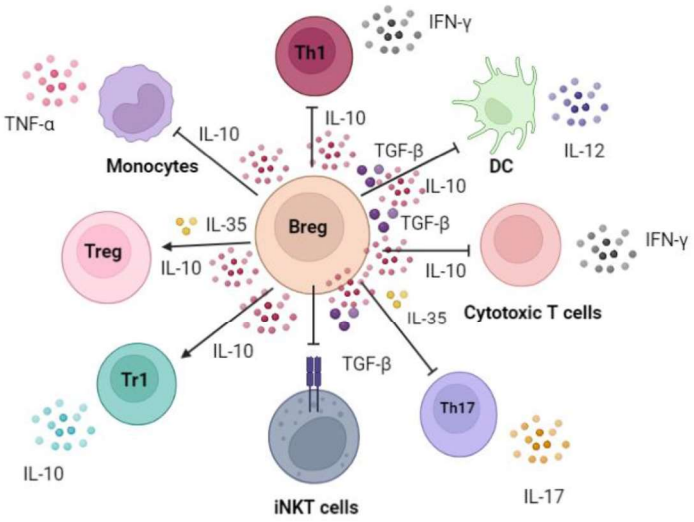


Figure 1.4: Suppressive molecules of Bregs & effects of Bregs on other immune cells (Adapted from Rosser et al,2015; Illustrated in BioRender).

1.4 Toll-Like Receptors

Toll-like receptors (TLRs) are one of the pattern recognition receptor (PRR) classes that recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) and they alert host cells to the presence of foreign invaders (Medzhitov et al., 1997). TLRs can induce both innate and antigen-specific adaptive responses. In addition to their roles in inflammatory responses; TLR signaling also mediates suppressive and protective immune responses in immunity (Mauri & Bosma, 2012).

There are 12 identified murine TLR members as TLR1-9, TLR11, TLR12, and TLR13, and 10 human-identified TLR members such as TLR 1-10 (Wang et al., 2016). TLRs are expressed on immune cells such as macrophages, eosinophils, dendritic cells (DCs), neutrophils, T cells, B cells, and some non-immune cells as epithelial or endothelial cells. All murine B cells express TLR 1-9.

TLRs can be located on the cell surfaces or intracellular compartments such as endosomes, endolysosomes, lysosomes, or ER. While TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are found in the cell surface; TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 are found in intracellular compartments (Kawasaki & Kawai, 2014). These receptors can bind to proteins such as flagellin; peptidoglycan and lipoteichoic acid (LTA) of Gram-positive bacteria; lipopeptides, lipomannans, or lipoglycans of mycobacteria; lipopolysaccharide (LPS) from Gram-negative bacteria;

double or single-stranded RNA or DNA from viruses or bacteria and zymosan from yeast. Some of the TLRs and their respective PAMPs or DAMPs can be seen in Figure 1.5 (El-Zayat et al., 2019). Toll-like receptors are made of two domains: Leucine-rich repeats (LRRs) containing ectodomain and cytoplasmic Toll/IL-1 receptor (TIR) domain. LRRs of ectodomains have horseshoe-like structures which interact with their particular PAMPs and DAMPs. Upon recognition of PAMPs or DAMPs by LRRs; TLRs interact with TIR domains containing adaptor proteins such as Myd88, TRIF, TIRAP, TRAM, and SARM to initiate signal transduction pathways (Akira & Takeda, 2004; Botos et al., 2011). As a result of downstream signaling, interferon regulatory factors (IRFs), MAP kinases, and NF-kB can be activated by IRAK1, IRAK2, IRAK4 or TRAF3, and TRAF6 for regulating the expression of cytokines, chemokines, and type 1 interferon (IFN) (Kawasaki & Kawai, 2014).

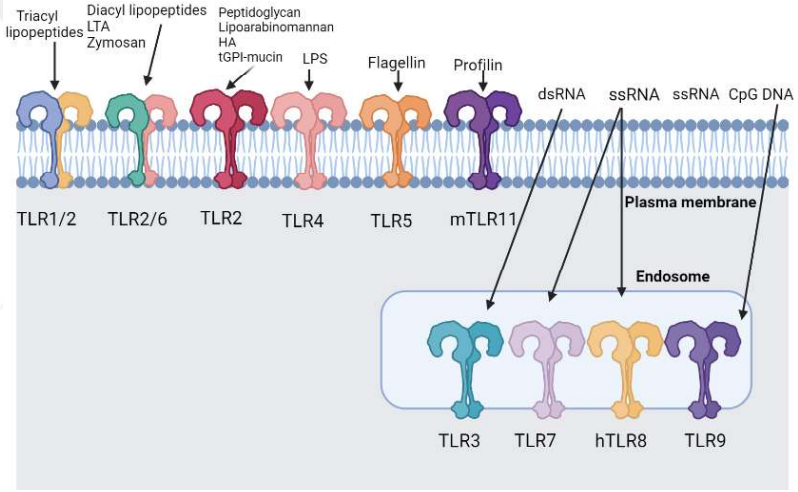


Figure 1.5: TLRs and their localization inside the cell (Adapted from El Zayat et al,2019;Illustrated in BioRender).

1.5 Mitochondria

Mitochondria are double membrane organelles that regulate energy metabolism in eukaryotic cells. In addition to energy production; mitochondria have different roles such as controlling signaling pathways and cell death, providing homeostasis for reactive oxygen species (ROS) and Ca^{+2}, and contributing to enzymatic reactions like steroid, pyridine, and heme synthesis; phospholipid modifications. These organelles

are found in all cell and organ types but their number and morphology are heterogeneous (Wang et al, 2017).

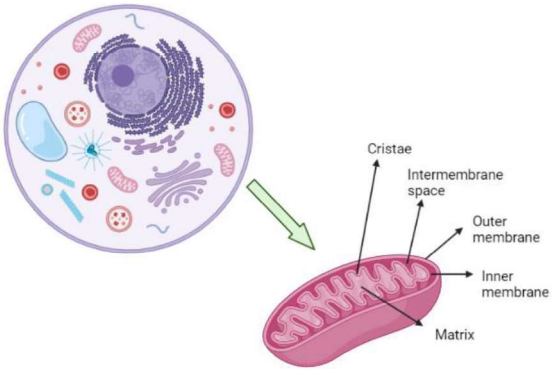


Figure 1.6: Structure of a mitochondrion (Illustrated in Bio Render).

In figure 1.6, the structure of a mitochondrion can be seen. There are two mitochondrial membranes: inner and outer. The outer mitochondrial membrane (OMM) directly links mitochondria to other molecules which are inside the cell. This makes OMM, a signaling platform for mitochondrial dynamics. Some anti-apoptotic proteins like Bcl-2, mitochondrial transporters TOMs, mitochondrial porins as VDAC, regulators of mitochondrial dynamics, and mitofusin proteins Mfn1 or Mfn2 are located on OMM (Breda et al., 2019). Inner mitochondrial membrane (IMM) is impermeable to ions and small molecules compared to OMM but it contains other membrane transporters and electron transport chain (ETC) compartments for energy production. IMM encloses a viscous consistency which is called a mitochondrial matrix. Ribosomes, soluble enzymes, nucleotides, and mtDNA are found in the mitochondrial matrix.

1.5.1 Mitochondrial DNA (mtDNA) and transcription factors

As mentioned above; mitochondria have their DNA which is located in the matrix. While the nuclear genome contains more than 1000 mitochondrial genes; mtDNA includes 37 mitochondrial genes. mtDNA encodes 22 genes for transfer RNAs, 2 ribosome RNAs, and 13 important oxidative phosphorylation polypeptides: ND1, ND2, ND3, ND4L, ND4, ND5, ND6 (parts of Complex I); Cytochrome b (parts of Complex III), COI, COII, COIII (parts of Complex IV) and ATP6, ATP8 (parts of Complex V) (Wang et al, 2017).

Studies have shown that oxidative signals, inflammation or oxidative stress, aging, or senescence can alter mtDNA. Although, it seems that mtDNA codes for a lesser

amount of mitochondrial proteins; mutation of genes can lead to deficiencies in energy production, organ damage, or dysfunction.

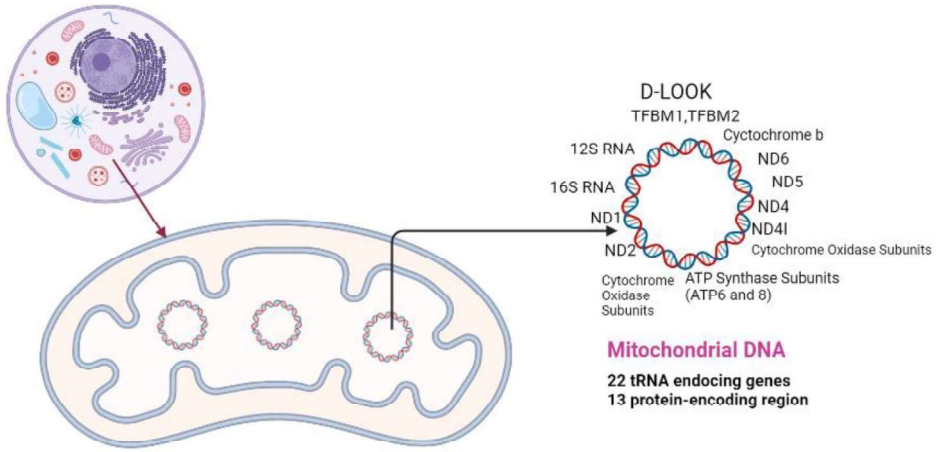


Figure 1.7: Mitochondrial DNA and its genes (Adapted from Wang et al, 2017;Illustrated in BioRender).

mtDNA transcription is controlled by mitochondrial transcription factor A (Tfam), mitochondrial RNA polymerase (POLRMT), and mitochondrial transcription factor B1 and B2 (Tfb1m and Tfb2m). Tfam binds to mtDNA and covers 14-35 bp upstream of the light strand promoter transcription initiation site and induces the assembly of the transcription initiation complex. Tfb1m is mitochondrial methyltransferase. It methylates 12srRNA and regulates the assembly of ribosome subunits and the expression of mitochondrial genes. Tfb2m facilitates the formation of the first phosphodiester bond and the structural changes of the transcription initiation site affecting both light strand promoter and heavy strand promoter 1.

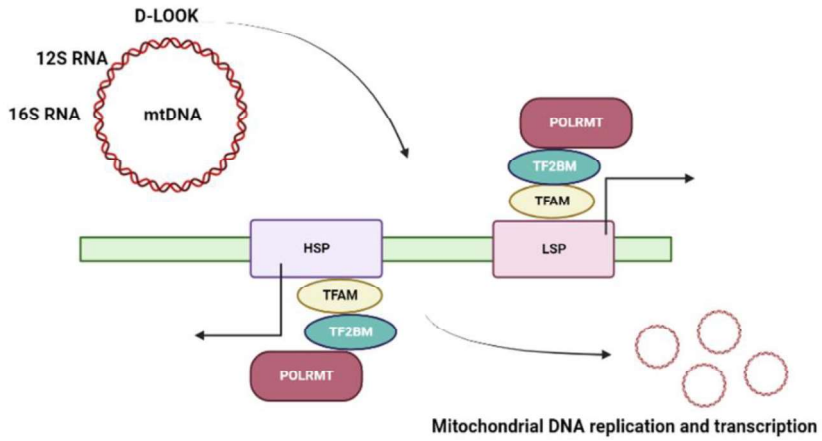


Figure 1.8: Mitochondrial transcription factors (LSP: Light strand promoter, HSP: Heavy strand promoter) (Adapted from Wang et al, 2017;Illustrated in BioRender).

1.5.2 Mitochondrial membrane potential (MMP)

During oxidative phosphorylation, redox transformations generate both electrical potential and proton gradient and together they form the transmembrane potential of hydrogen ions. This potential is called mitochondrial membrane potential (MMP or ΔΨm) and serves as an intermediate form of energy storage which is used by ATP synthase to make ATP. For the proper cellular functions, mitochondrial membrane potential (ΔΨm) needs to be at stable levels (Zorova et al., 2018). But in physiological activities such as activation or cellular damage, ΔΨm can be transient. In addition to ATP synthesis, ΔΨm can be used in the elimination of dysfunctional mitochondria. While high ΔΨm represents active and healthy mitochondria; low ΔΨm indicates dysfunctional mitochondria.

1.5.3 Mitochondrial biogenesis

Mitochondria have different important roles beyond energy generation because of this homeostasis of the mitochondria needs to be controlled for each healthy cell. Mitochondrial homeostasis comprises two fine-tuned processes: mitochondrial biogenesis and mitophagy. Mitochondrial biogenesis is the production of new and healthy mitochondria and results in increased oxidative phosphorylation capacity, repairment of mitochondrial-associated dysfunctions, and reduction of pathologic oxidative stress. Mitochondrial biogenesis involves mtDNA transcription and translation and mitochondrial protein synthesis, import, and assembly which is encoded by nuclear DNA (nDNA). Due to their roles in attending these biological roles peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α, nuclear respiratory factor-1(NRF-1), NRF-2, and estrogen-related receptor-α (ERR-α), mitochondrial transcription factor A (Tfam) are the master regulators of mitochondrial biogenesis (Popov, 2020). mtDNA copy numbers, the elevated mtDNA:nDNA ratio and the level of mitochondrial gene expression are the known mitochondrial biogenesis markers for all of the metabolism studies.

1.6 Energy Metabolism

Metabolism is the sum of all anabolic and catabolic reactions inside the cell which are respectively the production or breakdown of cellular substances. For the growth, differentiation, or survival of a cell; metabolism is regulated by both intracellular and extracellular signaling pathways (O'Neill et al., 2016). In mammalian cells, there are

six major metabolic pathways including glycolysis, tricarboxylic acid cycle (TCA), pentose phosphate pathway, fatty acid synthesis, fatty acid oxidation, and amino acid pathway (Hosomi & Kunisawa, 2020). From these pathways; glycolysis and oxidative phosphorylation are the ones that are responsible for energy production. In this thesis; mostly oxidative phosphorylation which comprises the both TCA cycle, and ETC reactions will be discussed.

1.6.1 Glycolysis

Glucose is one of the main sources of energy production. When glucose is present; it enters cells via glucose transporters and hexokinases convert it into glucose 6-phosphate. A sequence of enzymatic reactions called glycolysis taking place in the cytoplasm converts glucose 6-phosphate to pyruvate. During the formation of pyruvate; a total of 2 ATP and 2 $NADH_2$ is produced. Under an aerobic environment, pyruvate is converted to acetyl CoA with the release of CO_2 and $NADH_2$ cofactor molecules. Afterwards; acetyl CoA which is derived from pyruvate, enters the mitochondria for oxidative phosphorylation. If the environment is not aerobic; pyruvate will stay in the cytoplasm and be converted to lactic acid by lactate dehydrogenase (Chaudhry & Varacallo, 2021). But in some cell types such as cancer or immune cells; even under aerobic conditions glucose can be used for the generation of lactic acid. This is called "aerobic glycolysis" or "Warburg effect" (Ganeshan & Chawla, 2014).

1.6.2 Oxidative phosphorylation

In cells, most of the cellular energy is produced by oxidative phosphorylation (OXPHOS). It takes place in mitochondria and high energy is obtained from the breakdown of fats and carbohydrate molecules. In OXPHOS; electrons formed from the tricarboxylic acid cycle (TCA); combine with molecular oxygen (final acceptor of electron transport chain) and result in many oxidation/reduction reactions energy is released for the production of ATP from ADP. OXPHOS uses electrons from the TCA cycle for electron transport chain (ETC) reactions.

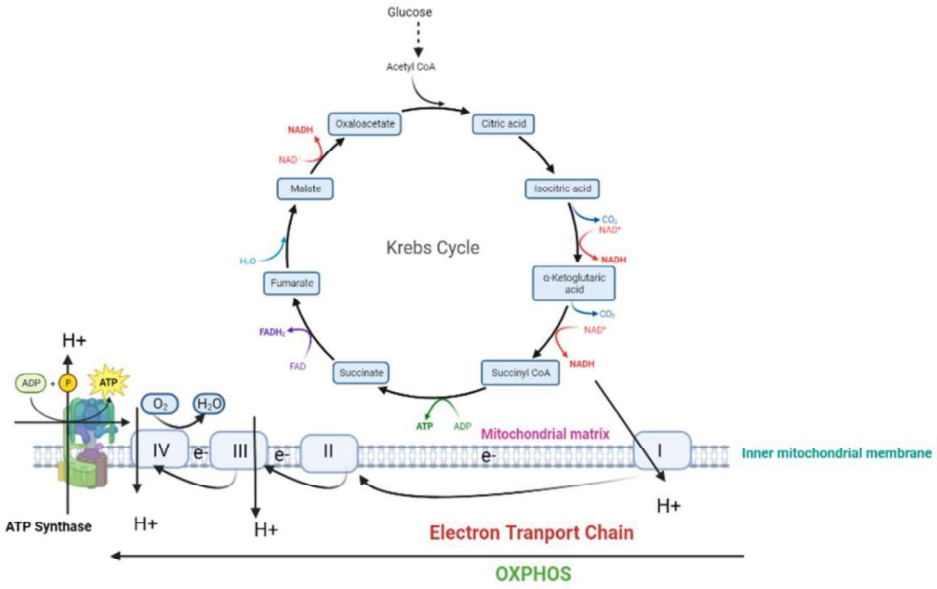


Figure 1.9: TCA cycle and OXPHOS are tightly coordinated (Adapted from Martínez-Reyes & Chandel, 2020; Illustrated in BioRender).

1.6.2.1 TCA cycle

TCA cycle is a sequence of enzymatic reactions in a closed loop that occurs in the mitochondrial matrix (Martínez-Reyes & Chandel, 2020; O'Neill et al., 2016). In 1937 Hans Adolf Krebs and his colleagues identified the TCA cycle and afterward, the cycle is called Krebs reactions.

The cycle begins with the production of citric acid from two-carbon acetyl CoA which is derived from pyruvate or fatty acids and four-carbon oxaloacetic acid (OAA). Because citric acid is the first product of the TCA cycle; reactions are also named as a citric acid cycle. Then, citric acid is converted into its six-carbon isomer isocitrate. Isocitrate is decarboxylated into five-carbon α-ketoglutarate and then four-carbon succinyl-CoA. During these reactions, two molecules of $NADH_2$ are produced when two molecules of CO_2 are released. Succinyl CoA then forms succinate with the production of GTP molecules. Next, succinate is converted into fumarate with the help of succinate dehydrogenase (SDH). During this enzymatic reaction, hydrogen atoms are added to FAD for producing $FADH_2$. Then fumarate is converted into malate and OAA which can react with another acetyl-CoA molecule to complete another TCA cycle reaction.

In addition to pyruvate which is formed during glycolysis, fatty acid oxidation is also a cellular source for the generation of acetyl CoA molecules. Even the first enzymatic reactions take place in the cytoplasm; beta-oxidation of fatty acids mostly occurs at

mitochondria and it yields cofactors such as $FADH_2$, and $NADH_2$ in addition to acetyl CoA. Produced acetyl CoA can be coupled with OAA by entering the TCA cycle to drive OXPHOS for producing a high amount of ATP (Talley & Mohiuddin, 2022).

1.6.2.2 Electron transport chain reactions

OXPHOS involves two different reactions for energy production: ETC reactions and chemiosmotic coupling. ETC comprises five protein complexes that can be found in the IMM and different organic molecules. For ATP generation, electrons need to transfer from $NADH_2$ or $FADH_2$ to oxygen but this is a very energy-yielding process with $\Delta G^{\circ\prime}$ = -52.5 kcal/mol for each pair of transferred electrons (Cooper et al.,2000). This energy should be produced gradually to be obtained in usable forms with the help of the passage of electrons through a sequence of carriers which are complex I, coenzyme Q (ubiquinone), complex III, cytochrome C, and complex IV (Ahmad et al., 2022).

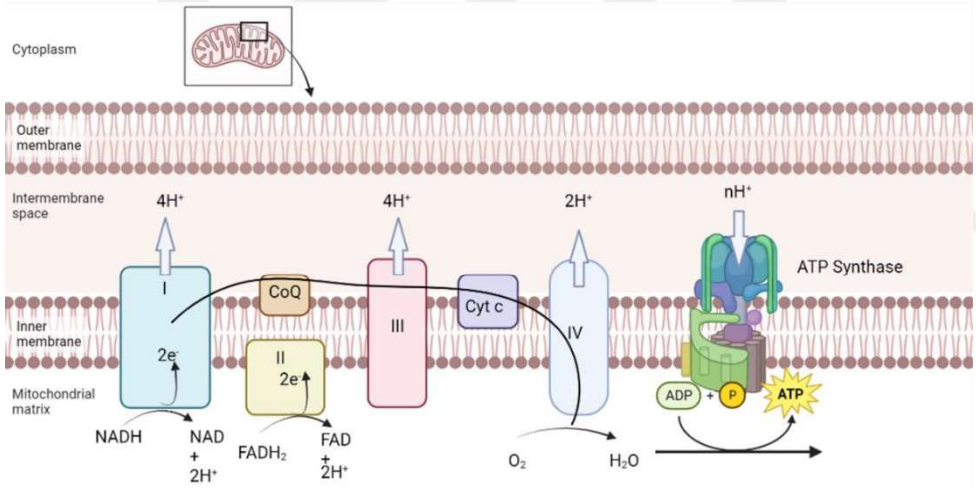


Figure 1.10: Electron transport chain reactions (Illustrated in Bio Render).

Electrons coming from $NADH_2$ enter the ETC first in complex I and move into coenzyme Q through flavin nucleotide and iron-sulfur carrier. Coenzyme Q is a lipid-soluble and small molecule and carries electrons from complex I to complex III. Cytochrome b is a part of complex III and is responsible for the transfer of electrons to cytochrome c. Cytochrome c carries electrons to complex IV where the electrons finally are transferred to the final acceptor O_2. In contrast to $NADH_2$, $FADH_2$ uses complex II. Then, respectively electrons are transferred into coenzyme Q, complex III, and complex IV.

1.6.2.3 Chemiosmotic coupling

The mechanism of coupling ATP production to electron transport is named chemiosmotic coupling. When electrons are transferred through complexes I, III, and IV; free energy from the electron transfer generates a proton gradient across the mitochondrial matrix to intermembrane space. Complex I and III pumps 4 H^+ but complex IV pumps 2 H^+ to intermembrane space. 2 other H^+ ions are pumped from complex IV; form water when it reacts with molecular oxygen in the matrix (In figure 1.10).

ATP synthase is also known as complex V and it has two subunits: F0 and F1. While F0 has hydrophobic properties and is buried into IMM, F1 is hydrophilic and oriented into the mitochondrial matrix. Together they generate a rotational motor system for energy production. F0 comprises a proton channel that gets protonated or deprotonated due to H^+ ions flowing through intermembrane space to the matrix. Changes in proton channels of F0's ionization results in rotation and changes in F1 subunit orientation. For each 4 H^+ ion which moves into complex V, 1 molecule of ATP is produced due to conformational changes in the F1 subunit because it catalyzes the production of ATP from ADP and Pi (Junge & Nelson, 2015; Okuno et al., 2011). After protons span F0 and ATP is produced, protons return to the mitochondrial matrix.

1.7 Immunometabolism

Two of the most critical features of multicellular life are metabolism and immunity and they are needed in order to distribute nutrients across cells, tissues & organs and to protect from injury and inflammation respectively (Makowski et al., 2020). The field of immunometabolism refers to the area which connects immune cell functions and intracellular metabolic pathways. There are six metabolic pathways inside the cells: glycolysis, the TCA cycle, fatty acid oxidation, the pentose phosphate pathway, fatty acid synthesis, and amino acid synthesis. Studies have shown that all of these pathways affect immune cell response via nutrient sensors and signal transduction by influencing their survival, differentiation, and function for obtaining organismal

homeostasis. In this thesis, mostly the relationship between mitochondrial metabolism and immunity will be explained and discussed.

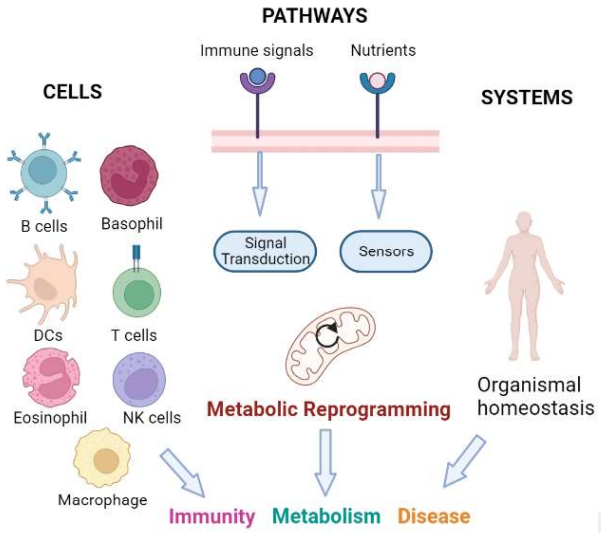


Figure 1.11: Relationship between immunity and metabolism (Adapted from Chi et al, 2022;Illustrated in BioRender).

1.7.1 Immunometabolism of B cell fate and function

Like other immune cells, metabolism also affects B cell development, activation, and function. During B cell development, hematopoietic stem cells undergo multiple differentiation steps of precursor cells such as pro-B, pre-B, and immature/transitional B cells for generating both B1 and B2 cells. While for B1 cells; glycolysis, lipid metabolism, and OXPHOS are necessary for the transition from pro-B cells to pre-B cells stage; for B2 cells mostly glycolysis controls transition in the PI3K-AKT-mTORC1 axis with AMPK activity. Immature B cells rely on glycolysis and OXPHOS but mature cells decrease these two metabolic pathways (Fu et al., 2022).

After receiving antigen, B cells get activated and change their metabolic demands. Different antigens (TI or TD antigens) affect different metabolic pathways to change B cell functions. For example, BCR stimulation increases aerobic glycolysis to generate lactic acid molecules. In contrast, proliferating B cells tend to skew toward the pentose phosphate pathway from glycolysis for producing 5-phosphate ribose and cofactor NADPH to synthesize nucleotides.

LPS-stimulated B cells use both glycolysis and OXPHOS respectively by enhancing glucose transporter 1 (GLUT1) expression to uptake glucose and mitochondrial mass but another study shows that LPS-stimulated Bregs upregulate the expression of ATP-citrate lyase to synthesize intracellular fatty acids such as cholesterol, free fatty acids,

neutral and acidic phospholipids for CD138, Blimp-1 expression, and proliferation of B cells. Plasma cells enhance glycolysis, expression of amino acid transporters, and their anabolic reactions such as lipid synthesis for producing antibody molecules and pyruvate-dependent mitochondrial functions to maintain their lifespan (Bibby et al., 2020; Caro-Maldonado et al., 2014).

Studies have shown that in addition to cholesterol metabolism Bregs increased their glycolytic flux by increasing HIF-1 alpha and this is accompanied by IL-10 producing CD11b $^{+}$ Bregs generation and vitamin D modulates calcium uptake to increase IL-10 expression. In contrast to glycolysis, the effect of the other metabolic pathways such as fatty acid metabolism, pentose phosphate pathway, and OXPHOS on the Breg and other B cell subsets differentiation and function needs to be elucidated (Heine et al., 2008; Waters et al., 2018).

1.7.2 Mitochondria and immunity

Mitochondria and immune response are closely linked to each other. Normally immune cells are metabolically quiescent. During immune responses, they transited to the active phase by shifting from the catabolic state to the anabolic state. In the quiescence state, cells catabolize macromolecules to produce energy and support cellular survival. In the anabolic state, cells synthesize macromolecules and need to balance required metabolites and ATP generation. Mostly, immune cells choose high-energy-producing pathways such as beta-oxidation to generate more ATP than glycolysis. Signals which are produced by both metabolic intermediates and ATP activate immune responses (Angajala et al., 2018). Because mitochondria are the main energy-producing organelle it plays important roles in maintaining and providing proper immune responses.

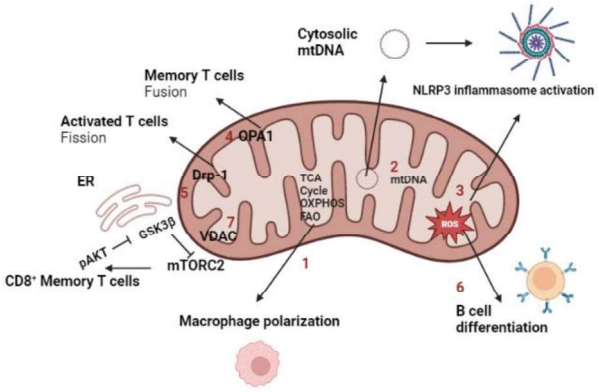


Figure 1.12: Immune cells affected by mitochondria (Adapted from (Angajala et al., 2018;Illustrated in BioRender).

Studies have shown that mitochondrial machinery components such as mitochondrial DNA (mtDNA), amino acid metabolism, mitochondrial reactive oxygen species (mtROS), Tfam, ATP, cardiolipins, MAVS, and other mitochondrial proteins induce immune responses in macrophages, T cells, eosinophils and dendritic cells (DCs) (Iwasaki et al., 2020). Also immune molecules such as cytokines; affect mitochondrial metabolism and vice versa while they can change different signaling pathways. For instance, in LPS-stimulated macrophages, IL-10 shifts metabolism from glycolysis to OXPHOS with the inhibition of the mTOR molecule (Ip et al., 2017).

Similar to other immune cells, studies have demonstrated that B cell activation with BCR or different TLR ligands changes mitochondrial dynamics. LPS-stimulated B cells enhanced mitochondrial mass at 6h time points (Caro-Maldonado et al., 2014), or co-stimulation of B cells with BCR ligand IgM and TLR9 ligand CpG increases mtDNA:nDNA ratio and Tfam expression as an indicator of mitochondrial biogenesis (Akkaya et al., 2018). For proper B cell differentiation and function mitochondria and their components such as transcription factors, mitochondrial proteins, and ROS plays a critical role. Mitochondrial ROS controls B cell fate decisions of CD40L-IL-4 or LPS-IL-4 stimulated B cells. After stimulation mitochondria generate ROS and the amount of ROS affects B cell differentiation together with mitochondrial membrane potential and mass. B cells with higher mass and membrane potential undergo class switch recombination, and B cells with moderate mass and membrane potential induce plasma cell differentiation (Jang et al., 2015). For secretion of antigen-specific antibodies from long-lived plasma cells mitochondrial pyruvate carrier 2, transporter of pyruvate from matrix to mitochondria is necessary. In mice lacking this mitochondrial protein; plasma cells' lifespans and antibody secretion were reduced (Lam et al., 2016). Even though these studies show that mitochondria play important roles for B cells, there is no clear evidence that elucidates the relationships between Breg cells and OXPHOS.

1.8 Aim of this study

H. pylori is a spiral-shaped, microaerophilic gram-negative bacterium. It can lead to gastro pathologies and more than 50% of the world population has been infected with *H. pylori*. *H. pylori* have less capacity to activate an efficient immune response in mice compared to *H. felis*. *H. felis* is used to generate mice models for studying this

pathogen. Because of this, during our mice experiments, *H. felis* antigen was used (Mégraud et al., 2015).

In addition to triggering immune responses; recent studies reveal that; TLR signaling can also affect the energy metabolism of B cells. TLR4 (LPS), and TLR9 (CpG) stimulated B cells to increase their glycolysis or oxidative phosphorylation (Akkaya et al., 2018; Caro-Maldonado et al., 2014; O'Neill et al., 2016). *H. felis* antigen is recognized by TLR2 on B cells and induces secretion of IL-10. Nevertheless, the energy metabolism of *Helicobacter*-stimulated B cells has not been elucidated yet. The main aim of this project is to investigate the mitochondrial metabolism of *Helicobacter felis*-activated B cells.

To serve this purpose; splenic B cells were isolated from C57BL/6 or IL-10 GFP reporter (VertX IL10 ^{egfp}) mice and stimulated with *Helicobacter felis* sonicate. Then, at corresponding time points; *H. felis* -activated B cells are used for mitochondrial mass and membrane potential assays to check if these cells have functional mitochondria or not. Afterwards, for understanding mitochondrial DNA number or mitochondrial biogenesis; mtDNA:nDNA ratio and mitochondrial transcription factor A (Tfam) expression level in *H. felis*-activated B cells were analyzed respectively.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacteria

Helicobacter felis was supported by Prof. Dr. Anne Müller from the University of Zurich. Bacteria were cultured on a Columbia Agar plate containing a 1000 X antibiotic cocktail. Contents of the Columbia Agar medium was prepared as shown in Table 2.1.

Table 2.1: The components of Columbia Agar Medium.

Component	Amount
BD Columbia Agar	42,5 g
Horse Blood	50 ml
β-cyclodextrin	10 ml
1000X Antibiotic Cocktail	1 ml

The components of 1000x Antibiotic cocktail were given in Table 2.2.

Table 2.2: The components of 1000x Antibiotic Cocktail.

Component	Amount	Company
Trimethoprim	100 mg	HiMedia
Amphotericin B	160 mg	Genaxxon
DMSO	20 ml	Genaxxon

2.1.2 Liquid culture for *Helicobacter felis*

For the preparation of liquid culture of *H.felis* Brucella Broth was used. Ingredients of liquid culture were given in Table 2.3.

Table 2.3: The components of liquid medium for *H.felis*.

Component	Amount	Company
Brucella Broth	50 ml	HiMedia
FBS 10% (v/v)	5 ml	Euroclone
Vancomycin	5 µl	HiMedia

2.1.3 Long term storage of *Helicobacter felis*

Helicobacter felis freezing medium was prepared by using the contents that were given in Table 2.4. When prepared, the freezing medium was stored at +4 °C.

Table 2.4: The contents of Freezing Medium for *H.felis*.

Constituent	Amount
Brucella Broth	25 ml
Glycerol	25 ml

2.1.4 Primary cells culture

CD19 B^+ cells obtained from the spleens of C57BL/6 mice or IL-10 GFP reporter (VertX IL10 ^{egfp}) with magnetic separation using Midi Macs separator (Miltenyi). Isolated B cells were cultured in a complete RPMI medium containing 55 μM 2-ME, 1% Penicillin-Streptomycin, 10 % FBS and 25mM HEPES. Cells were stimulated with 10 μg/ml *Helicobacter felis* sonicate, 2.5 μg/ml PAM3CSK4, and 10 μg/ml lipopolysaccharide (LPS).

Table 2.5: Cell culture reagents.

Solution	Company
Roswell Park Memorial Institute Medium (RPMI)	Sigma
Fetal Bovine Serum (FBS)	Euroclone
Penicillin-Streptomycin	Gibco
1M HEPES Buffer	Capricorn
β-Mercaptoethanol	Sigma
Trypan blue	Lonza

Preparation of solutions used in cell culture studies in cell culture were given in Table 2.6.

Table 2.6: Preparation of solutions used in cell culture studies.

Buffer	Preparation
PBS 1 X	9,55 g dPBS in 1 liter of ddH20
MACS Buffer	0.5 % BSA and 2 mM EDTA in 1x PBS
FACS Buffer	2% FBS in 1 X PBS
Complete RPMI	10% FBS, 1% Penicillin-Streptomycin, 25mM HEPES, 55 μM 2-ME in incomplete RPMI

2.1.5 IL-10 ELISA solutions and standards

2.1.5.1 Solutions

The solutions used in the ELISA experiment were given in table 2.7.

Table 2.7: Solutions used in ELISA

Solution	Preparation
PBS/T (0.05 %)	0.05 % Tween in 1 X PBS
Stop Solution	2N H_2SO_4 (in ddH_2O)

2.1.5.2 IL-10 standards

Standards of IL-10 were prepared as kit's instructions. 1X Assay Diluent A was used as diluent. First, the 10 ng lyophilized IL-10 standard was reconstituted with 200 µl 1x Assay Diluent A and aliquoted as 40 µl for obtaining 50ng/ml IL-10 five stock solutions. For each assay, 960 µl 1x Assay Diluent A was added into one of the 50 ng/ml IL-10 stock to prepare 2000 pg/ml top standard. After that, seven two-fold serial dilutions of the 2000 pg/ml were performed to obtain mouse IL-10 standard concentrations: 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.25 pg/ml, 15.625 pg/ml. As a blank solution, 1x Assay Diluent A was used.

2.1.5 BCA assay standards

Standards of BCA assay were prepared as kit's instructions. ddH_2O was used as diluent. The components and their volumes of BCA assay standards were listed in Table 2.8.

Table 2.8: Preparation of BSA standards for BCA Assay.

Standards	Diluents (µl)	BSA(µl)	Final BSA Concentration (µg/µl)
A	0	300 µl from stock	2000
B	125	375 µl from stock	1500
C	325	325 µl from stock	1000
D	175	175 µl from stock	750
E	325	325 µl from stock	500
F	325	325 µl from stock	250
G	325	325 µl from stock	125
H	400	100 µl from stock	25
I	400	0	0=Blank

2.1.6 Commercial kits

Kits used in this study are listed with their supplier company in the table 2.9.

Table 2.9: Commercial kits used in the experiments.

Kit	Company
Mouse B cell isolation kit	Miltenyi Biotech.
Mouse IL-10 Deluxe Max ELISA Kit	Biolegend
BCA Protein Assay Kit	Thermo Scientific
PureLink Genomic DNA Isolation Kit	Thermo Scientific
Real Q Master Mix	Amplicon

2.1.7 General Chemicals

Chemicals used in the experiments were given in Table 2.10.

Table 2.10: Chemicals used in the experiments.

Chemical	Company
EDTA	Sigma Aldrich
Absolute ethanol	Isolab
Phosphate Buffer Saline (PBS)	Cegrogen
Bovine Serum Albumin (BSA)	HiMedia
Cell culture grade-DMSO	Genaxxon
β-Mercaptoethanol	Sigma Aldrich
PAM3CSK4	Invivogen
Lipopolysaccharide (LPS)	Sigma Aldrich
Tween 20	Biomatic
Fixation Buffer (4%)	Biolegend
Permeabilization Buffer (10x)	Biolegend
Sulfric Acid (H_2SO_4)	Fluka
Xtrazol Reagent	Biofroxx
FCCP	Abcam

2.1.8 Laboratory equipment and materials

Laboratory equipment and materials used in the experiments were given in Table 2.11 and Table 2.12 respectively.

Table 2.11: List of the laboratory equipment used in the experiments.

Equipment	Supplier Company
Laminar Air Flow Cabinets	FASTER BH-EN 2003
Flow cytometer	BD Accuri C6
Step One Real-Tiem PCR	Applied Biosystem
Weight	Precisa
Nanodrop 2000	Thermo Scientific
Spectrophotometer	BIO-RAD Benchmark Plus
Refrigerators	Profilo (+4 C) Siemens (-20 C) Arçelik(-20 C)
Electronic Pipette	CappAid Labware
Pipettes	2.5µl,10 µl, 200 µl, 1000 µl Eppendorf ,10 µl, 20 µl, 100 µl, 200 µl, 1000 µl Socorex and 10 µl, 100 µl, 1000 µl Biohit
Centrifuges	Beckman Coulter Allegra ™ 25 R Centrifuge Scanspeed 1730 R Labogene Scanspeed mini
CO2 Incubator	BINDER
Shaker	Heidolp Duomax 1030
Sonicator	Bandelin Sonopuls
Vortex	Mixer Uzusio VTX-3000L, LMS
Magnetic Stirrer	WiseStir MSH-20D, Wisd Laboratory Equipment
Light Microscope	Olympus CH30
Hematocytometer	Isolab
Ice Machine	Scotman AF10

List of materials used in the experiments were given in Table 2.12.

Table 2.12: List of material used in the experiments.

Material	Supplier Company
Gloves	Benchmark
Erlenmeyers	Isolab
Anaerobic Jar	Anaerocult
Falcon Tubes (15ml, 50 ml)	Labmarker
Slide	Interlab
Coverglass	Interlab
Cotton Swab	Interlab
96-well plate for ELISA	Nunc
Cell Strainer (70 μm)	Falcon
Serological pipette	Capp
96 well plates	TPP
Eppendorf tubes (0.5 ml, 1.5 ml, 2 ml)	Labmarker
LS Columns for Magnetic Separation)	Miltenyi Biotec
CampyGen 2.5L	Oxoid

2.1.9 Primers

Primers which were used in the PCR experiments and their properties were given in the Table 2.13.

Table 2.13: Primers which are used in the experiments.

Gene/bp	Primers	Species	Tm (C)
Cox1 116 bp	F: GCCCCAGATATAGCATTCCC R: GTTCATCCTGTTCCTGCTCC	Mouse	56
Rps18 352 bp	F: AGCCTGGACACCTCTTAGGAT R:CCCGTGTTCTTTCAGTCTCTAGT	Mouse	56
Tfam 260 bp	F: CCAAAAAGACCTCGTTCAGC R: ATGTCTCCGGATCGTTTCAC	Mouse	58
B2M 126 bp	F: GAGAATGGGAAGCCGAACATA R: GCTGAAGGACATATCTGACAT	Mouse	58

2.1.10 Antibodies and dyes

Antibodies or dyes used in this research are given in Table 2.14.

Table 2.14: List of antibodies or dyes which used in experiments.

Antibody/Dye	Clone	Supplier Company	Applications
7-AAD Staining Dye	-	Biolegend	FC
Mitoview Green	-	Biotium	FC
Mitoview 633	-	Biotium	FC
MitoStatus TMRE	-	BD	FC
CD19-PE	6D5	Biolegend	FC
TruStain fcX™ (anti-mouse CD16/32)	-	Biolegend	FC

2.1.11 Preparation of mitochondrial dyes

In order to prepare 200 μM stock solution of Mitoview Green and Mitoview 633, 50 μg vial of lyophilized dyes were dissolved with 400 μl and 460 μl cell-culture grade DMSO respectively. For preparing 1mM MitoStatus TMRE stock solution, 1 mg of TMRE was dissolved with 1.94 ml of cell-culture grade DMSO.

Mitochondrial oxidative phosphorylation uncoupler Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was used as a negative control of mitochondrial membrane potential assays. A 5 mM stock solution of FCCP was prepared by dissolving 1 mg of FCCP with 0.79 ml of cell-culture grade DMSO.

2.2 Methods

2.2.1 *H.felis* culture

H. felis was cultured on Columbia Agar plates containing an appropriate amount of antibiotics as Trimethoprim and Amphotericin B. The plates were incubated at 37°C in microaerophilic conditions in an aerobic jar for 3-4 days. The anaerobic microenvironment of the jar was provided by using CampyGen packets. Columbia blood agar was prepared by dissolving 42.5 g of Columbia Agar in 1 liter of distilled water and it was autoclaved. Then agar was incubated at 56 °C for 1 hour and after incubation, 50 ml of the horse blood was added to the bottle. 10 ml of β-cyclodextrin and 1 ml 1000x Antibiotic Cocktail were added to the agar solution for the growth of the bacteria. After 3-4 days of incubation; cell viability and motility were checked under the light microscope. Grown bacteria were transferred into Brucella Broth medium which contains 10 μg/ml of Vancomycin with the appropriate dilution rate. Brucella Broth medium, 28 g Brucella Broth powder was dissolved in 1 liter of distilled water and the liquid solution was sterilized by autoclaving it at 121°C for 15 min.

2.2.2 Sonication of *H. felis*

When *H. felis* liquid culture reached 120-200 ml, bacteria viability, and motility was checked under light microscopy. Then cultures were shared in 15 ml falcon tubes and falcons were centrifuged at 1500 rpm for 10 minutes. Supernatants were discarded and pellets were resuspended with 10 ml of 1x PBS for washing off the bacteria. Tubes

were centrifuged again at 1500 rpm for 10 minutes. The supernatant was discarded and the pellet was dissolved by using 3.5 ml PBS. The sonication procedure was initiated by setting the sonicator 30 seconds pulse on and 50 seconds pulse off for 6.5 min at 50 watts. Then a falcon tube was placed into the sonicator, and sonication was performed on ice to prevent overheating. For sonication, MS 72 probe was used. Following sonication; *H. felis* sonicates were aliquoted into 1.5 ml eppendorf tubes as 100 µl for each tube and tubes were centrifuged at 15000 for 20 minutes at 4°C. Supernatants were transferred to 0.5 ml Eppendorf tubes and stored at 4°C *H. felis* sonicate concentration was measured by BCA assay.

2.2.3 Protein bicinchoninic acid (BCA) assay

When sonication is completed, the concentration of *H. felis* sonicates was checked by using Thermo Scientific's Pierce BCA Assay Kit. First, bovine serum albumin standard sets were prepared as mentioned in Table 2.8. Then, solutions A and B were mixed in a 1:50 ratio according to standards and samples in single. The mixture of solutions A and B were added to the respective wells in a 96-well flat bottom plate as 200 µl for each well as an indicator for the assay. 10 µl samples and diluted BSA standards were put into the respective wells and then the plate was incubated at 37°C for 30 minutes. When the plate was cooled to room temperature, absorbance values of samples and standards were measured at 562 nm using a microplate reader.

2.2.4 Isolation of mouse B cells

B cells were isolated from the spleen of C57BL/6 mice or or IL-10 GFP reporter (VertX IL10 ^{egfp}) mice using the Mouse B cell Isolation Kit (MACS, Miltenyi). The isolation protocol is explained in detail in the following sections.

2.2.4.1 Preparation of single cell suspension

The spleen was obtained from a sacrificed C57BL/6 or IL-10 GFP reporter (VertX IL10 ^{egfp}) mouse. 70 µm cell strainer was put on a 50 ml falcon tube. The cell strainer was soaked with incomplete RPMI medium, and the spleen was transferred to the strainer. The spleen was smushed using the plunger of a syringe and the single-cell suspension was filtered into 50 ml falcon. The cell strainer again was rinsed using incomplete RPMI. These steps were performed for all the obtained spleens. Then, the falcon tube was filled with incomplete RPMI, and the cell suspension was centrifuged at 1200 rpm for 8 min. The supernatant was discarded and the cell pellet was dissolved

in 1 ml MACS buffer. Cell number was detected by diluting splenocytes with MACS buffer (1:100). Cell viability was determined with trypan blue staining.

2.2.4.2 Magnetic labeling of non-B cells

After cell viability and number were checked; cell suspension was centrifuged at 1200 rpm for 8 min. The supernatant was discarded and the pellet was resuspended in 40 µl of MACS buffer per 10^7 cells. Then 7 µl Biotin-Antibody cocktail was added onto cell suspension per 10^7 cells and incubated at 4 °C refrigerator for 5 min. 30 µl of MACS buffer and 14 µl Anti-Biotin Cocktail for 10^7 cells were added to the cell suspension and cells were incubated at 4°C refrigerators for 10 min.

2.2.4.3 Magnetic separation: Depletion of non-B cells

LS Column was placed to the Midi MACS magnetic separator. The column was activated by washing it with 3 ml of cold MACS buffer. After activation of the column, the cell suspension passed through the column. LS column was washed twice by 3 ml MACS buffer. The cells passing through the column were B cells; while the cells which were bound to the column were non-B cells. To acquire non-B cells LS column was transferred to a 15 ml falcon tube. 3 ml MACS buffer was added to the column and non-B cells were collected in this tube by strongly pushing the plunger into the column. Both B and non-B cell numbers were checked by a hematocytometer. For cell counting, cells were diluted with MACS buffer (1:100). Cell viability was checked with 7-AAD by using flow cytometry.

2.2.4.4 Determination of B cell purity

To determine freshly isolated B splenic cells, a flow cytometer was used. 10^5 B cells were stained with 0.5 µl PE-conjugated anti-CD19 antibody in 50 µl FACS buffer in the dark at 4°C for 30-45 min. B cells were dissolved in 200 µl FACS buffer without stain and were used for unstained control. After incubation, both stained and unstained B cells were centrifuged at 3000 rpm at 4°C for 8 minutes. The supernatant was discarded and pellets were dissolved with 200 µl FACS buffer. Cells were rapidly analyzed by a flow cytometer.

2.2.5 In vitro stimulation and cell culture

Isolated $CD19^+$ B cells were centrifuged at 1200 rpm for 8 min and cell pellets were dissolved at 1ml complete RPMI per $2.5x10^6$ B cells. Afterward, B cells were seeded

into 96 well U bottom plates as 150.000 cells for each well. For stimulation, cells were incubated with *H. felis* (10 µg/ml), PAM3CSK4 (2.5 µg/ml) and LPS (10 µg/ml) for 6,24 and 48 hours. Unstimulated B cells were used as a control group.

2.2.6 IL-10 ELISA

Biolegend's Mouse IL-10 Deluxe Max Kit was used to determine IL-10 protein levels in supernatants of samples. First, Nunc Maxisorp 96 well plate was coated with IL-10 capture antibody 1:200 diluted in 1x Coating Buffer B which was also diluted from 5x to 1x with molecular grade water in an appropriate falcon tube. The plate was sealed by using parafilm and incubated at +4°C overnight. The plate was washed four times with 1x PBS/T solution following the day. Assay Diluent A was diluted from 5x to 1x using 1x PBS solution. 100 µl of 1x Assay Diluent A was added to each well for preventing non-specific bindings. The plate was incubated at RT for 1 hour with shaking. Recombinant IL-10 standard proteins were prepared by serial dilutions according to the manufacturer's instructions as mentioned above. After incubation with Assay Diluent A, the plate was washed four times with PBS/T solution. 75 µl of standards and samples were loaded in respective wells as duplicates and the plate was incubated at +4°C overnight. Subsequently, the plate was washed four times with PBS/T solution. 200x biotinylated IL-10 detection antibody which was diluted to 1x with 1x Assay Diluent A solution, applied to each well and the plate was incubated at RT for 1 hour with shaking. The plate was washed four times with PBS/T and 1X Avidin-HRP (was diluted from 1000x with 1x Assay Diluent A) was added to each well. The plate was incubated at RT for 30 min with shaking. After incubation, the plate was washed six times with the final soaking step by using PBS/T. 50 µL of TMB substrate solution mixture (1:1 of TMB Substrate A and TMB Substrate B) was added to each well. The plate was incubated at RT in dark for 45 min- 1hour. TMB solution's color was changed to a blue color in the positive wells by the HRP enzyme. 50 µl of 2N H_2SO_4 Stop solution was added to each well and the reaction was stopped. Wells were turned yellow. Finally, absorbance values of samples and diluted IL-10 standard were measured at 450 nm on a microplate reader.

2.2.7 Flow cytometry

Cultured B cells were collected in 1.5 ml eppendorf tubes. Cells were centrifuged at 3000 rpm at 4°C for 8 min. Supernatants were transferred into 0.5 ml eppendorf tubes

and stored at -20 °C. Cell pellets were resuspended with 200 µl FACS buffer and centrifuged at 3000 rpm at 4°C for 8 min. The supernatant was discarded. Pellets resuspended with 50 µl pre-warmed mitochondrial staining buffer. Mitochondrial staining buffer includes 80 nM Mitoview Green for mitochondrial mass staining and 40 nM Mitoview 633 or 30 nM Mito Status TMRE for mitochondrial membrane potential staining in pre-warmed FACS Buffer. Mitoview Green dye is non-fluorescent until it partitions into the mitochondrial membrane and after it enters the cell, its signal on 488 nm is proportional to mass and volume of mitochondria. Mitoview 633 and TMRE are the mitochondrial membrane potential dyes and they signal with their binding to released H^+ ions of electron transport chain reactions. In addition to stained B cells; unstained B cells separated and dissolved in FACS buffer without any mitochondria dye. Cells were incubated at 37°C for 1 hour. After incubation, 150 µl pre-warmed FACS buffer was added to each tube, and cells were centrifuged. The supernatant was discarded. Cells were stained with 0.5 µl 7-AAD in 50 µl FACS buffer and incubated for 10 min at RT. Then 150 µl FACS buffer was added to each tube and cells were rapidly analyzed in the flow cytometer.

2.2.8 Gene expression studies

2.2.8.1 DNA isolation

For the analysis of mtDNA:nDNA ratio on Q-PCR, DNA isolation was made from cultured B cells using Thermo Scientific's PureLink Genomic DNA Isolation Kit. First B cell pellets were resuspended with 200 µl of cold 1X filtered PBS. Then 20 µL Proteinase K and 20 µl Rnase A were added to each sample for lysis of the cells and digestion of RNA respectively. Tubes were briefly vortexed and incubated at RT for 2 minutes. After incubation for each well 200 µl PureLinkR, Genomic Lysis/Binding Buffer was added and samples were incubated at 55°C for 10 minutes by using a heat block for promoting protein digestion. Then 200 µl of 96-100% ethanol was added to the lysate and mixed well by pipetting to yield a homogeneous solution. The lysate was added to PureLinkR Spin Column in a Collection tube and the column was centrifuged at 10.000 xg for 1 min at RT. The collection tube was discarded and the spin column was placed into a clean PureLinkR Collection Tube supplied with the kit. 500 µl Wash Buffer 1 was added to the column and the column was centrifuged at 10.000 xg for 1 min at RT. The collection tube was discarded and the spin column again was placed into a new, clean Collection tube. 500 µl Wash Buffer 2 was added

to the spin column and the column was centrifuged at maximum speed for 3 minutes at RT. The collection tube was discarded and the spin column was transferred into a 1.5 ml Eppendorf tube. 25 µl PureLink Genomic Elution Buffer was added into the column and columns were incubated for 1 min. Columns were centrifuged at maximum speed for 1 minute at RT. The spin column was discarded. The 1.5 ml Eppendorf tubes contain purified genomic DNA for each sample. DNA samples were stored at -20°C. DNA concentrations were determined using Nanodrop.

2.2.8.2 RNA isolation

In order to determine Tfam gene expression on RT-PCR, RNA isolation was made from cultured B cells. Cultured B cells were washed with 1x cold PBS. After the washing step, they were frozen as either cell pellets or resuspended with 1 ml Xtrazol Reagent and stored at -80°C. Stored samples were incubated for 5 minutes at RT. 0.2 ml of chloroform per 1 ml of Xtrazol reagent was added to each sample and pellets were resuspended via a 1 ml insulin syringe. Samples were incubated for 3-10 minutes at RT. After incubation samples were centrifuged at 12000 x g for 15 minutes at +4°C. In this step; the sample will be separated into a pale-yellow organic phase, interphase, and a colorless upper aqueous phase that involves the RNA. The aqueous phase was transferred to a new eppendorf tube and RNA was precipitated by mixing with 0.5 ml of cold 2-propanol per 1 ml of Xtrazol reagent. Samples were incubated for 10 minutes at RT. After incubation, samples were centrifuged at 12000 x g for 10 minutes at +4°C. The supernatant was removed and the RNA pellet was washed twice with 1 ml of 75% ethanol per 1 ml of Xtrazol reagent. The samples were mixed by using a vortex and centrifuged at 7500 x g for 5 minutes at +4°C. The supernatants were discarded and pellets were air dried. The RNA pellet was dissolved in the proper amount of RNAse free-water by pipetting. RNA concentrations were determined using Nanodrop. Then, RNA samples were stored at -80°C.

2.2.8.3 Conventional PCR

Conventional PCR was performed by using NEB's Taq PCR Kit. The reaction mixture was prepared according to the manufacturer's instructions. The volume of the components and thermal cycler conditions of the reactions were given in Table 2.15 and Table 2.16.

Table 2.15: Components of Conventional PCR reaction.

Components	Reaction Volume
Taq Buffer	2.5 µl
dNTPs (10 mM)	0.5 µl
Taq DNA Polymerase	0.125 µl
Forward Primer (10 µM)	0.25 µl
Reverse Primer (10 µM)	0.25 µl
Template DNA	2.5 µl
Molecular Grade Water	Up to 25 µl
	Total volume:25 µl

Table 2.16: Thermal cycler conditions of Conventional PCR reaction.

Step	Temperature	Time
Initial Denaturation	95°C	30 seconds
Cycling Stage (40 cycles)	95°C	30 seconds
	Depending on primers	30 seconds
	68°C	1 minute
Final Extension	72°C	5 minutes
Holding	4°C	∞

2.2.8.4 Real Time Q- PCR (RT-QPCR)

To analyze mtDNA:nDNA ratio and determine relative expression levels of TFAM in B cells, real-time PCR was carried out by using Real Q Master Mix. For normalization of the Tfam expression, beta-2 microglobulin housekeeping gene and for identifying of the mtDNA:nDNA; RPS18 housekeeping gene were used in this experiment. Obtained Ct values analyzed by delta-delta Ct method in using Excel and statistical analysis was done on GraphPad Prism. The volume of components and the conditions of the PCR reaction was given in Table 2.17 and Table 2.18.

Table 2.17: Components of RT-QPCR reaction.

Components	Reaction Volume
Real Q Plus 2x Master Mix	5 µl
Forward Primer (10 µM)	0.25 µl
Reverse Primer (10 µM)	0.25 µl
Template DNA	2.5 µl
Molecular Grade Water	Up to 10 µl
	Total volume:10 µl

Table 2.18: Conditions of RT-QPCR reaction.

Step	Temperature	Time
Initial Denaturation	95°C	15 minutes
Cycling Stage (40 cycles)	95°C	30 seconds
	Depending on primers	30 seconds
	72°C	30 seconds
Melting Curve	95°C	15 seconds
	Depending on primers	1 minute
	68°C	15 seconds
Holding	72°C	5 minutes

2.2.9 Analysis for flow cytometry

The results were analyzed by using FlowJo 10.8.1 software. Firstly, lymphocytes and single cell gates were taken for determining B cell populations. For identifying pure and dead B cells at 0h time point ; $CD19^+$ B cell and 7-AAD^+ B cell gates were taken inside the single cell population gate compared to unstained control group and values analyzed as percentages. At 6h, 24h and 48h time points; 7-AAD negative B cell gate was selected inside the single cell population gate for analyzing mitochondrial stainings inside the alive B cell populations. Histogram analysis of Mitoview Green, Mitoview 633 or MitoStatus TMRE dyes was evaluated in 7-AAD negative B cells and Mean Fluorescence Intensity (MFI) values were calculated and used in statistical analysis of mitochondrial mass of membrane potential experiments.

2.2.10 Statistical analysis

Graphpad Prism 8.0 was used for the calculation of p values and significance was determined by an unpaired two-tailed Student t-test. $p<0.05$ was considered statistically significant.

3. RESULTS

3.1 Splenic B cells were isolated from C57BL/6 mice with high purity levels.

B cells were isolated from the spleen of C57BL/6 or IL-10 GFP reporter (VertX IL10 ^{egfp}) mice using magnetic-activated cell sorting (MACS) negative selection. After isolation, the purity of the splenic B cells was determined by B cell-specific surface marker CD19 by flow cytometry. Figure 3.1A shows a representative flow staining of $CD19^{+}$ purified B cells compared to unstained B cells. Isolated and cultured B cell purity was higher than 97%. In addition to purity, the viability of splenic B cells was also evaluated by flow cytometry via 7-AAD viability staining. Figure 3.1B shows a representative flow staining of 7-AAD. The viability of purified B cells was higher than 96 % compared to the unstained control.

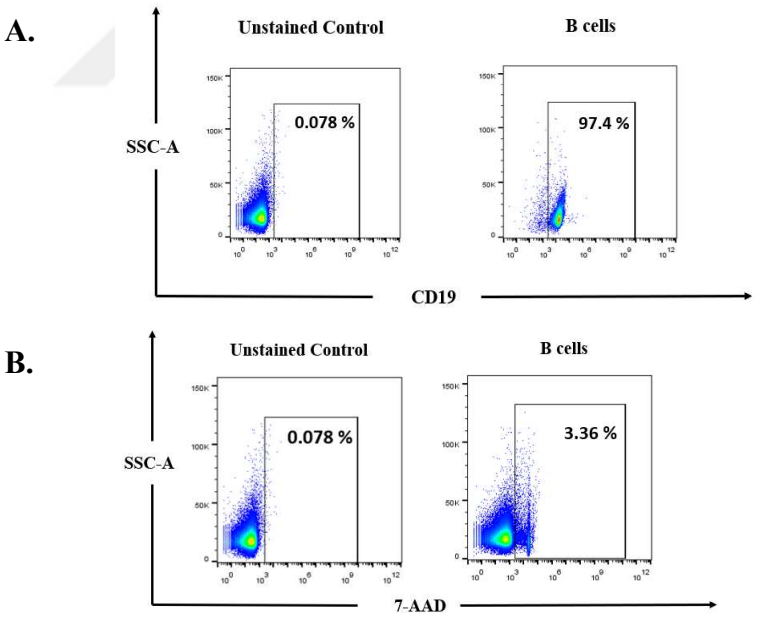


Figure 3.1: Purity and viability of isolated B cells. Representative flow cytometry plot of purified B cells compared to unstained control. B cells were isolated from the spleen of C57BL/6 mice by negative magnetic isolation. Isolated 10^4 B cells were either stained with PE-coupled anti-CD19 antibody or left unstained in order to detect the percentage of cells expressing B cell-specific surface marker CD19. The viability of purified B cells was determined via 7-AAD viability staining . Values written within quadrants indicate percentages of pure B cells (A) and dead cells (B).

3.2 IL-10 Secretion from *H. felis*, PAM3CSK4, and LPS Stimulated-B Cells was enhanced compared to the unstimulated group.

The IL-10 secretion levels of *H.felis*, PAM3CSK4, and LPS stimulated- B cells were determined by IL-10 ELISA at 6h, 24h, and 48h time periods. While at the 6h time, there are no significant changes in IL-10 levels between control and stimulated groups; IL-10 secretion was increased in *H.felis*, PAM3CSK4, and LPS-stimulated B cells at 24h and 48h time points compared to the unstimulated control group.

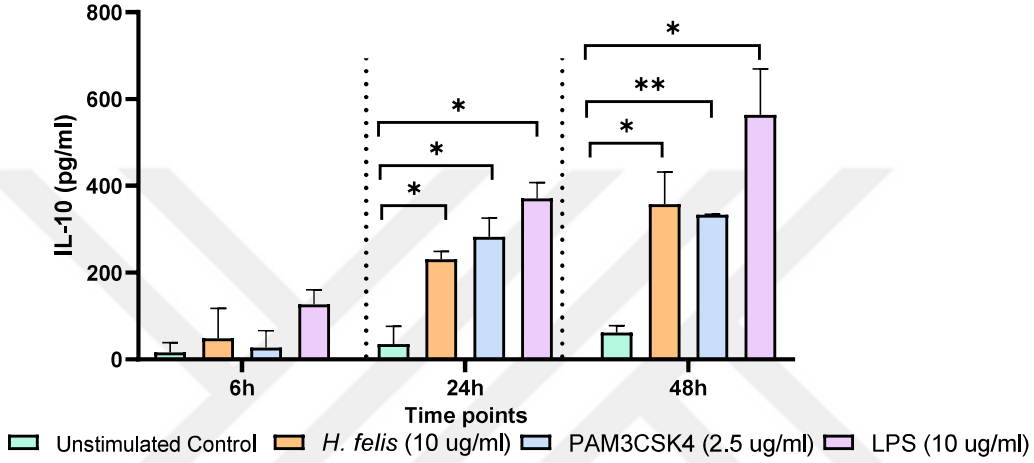


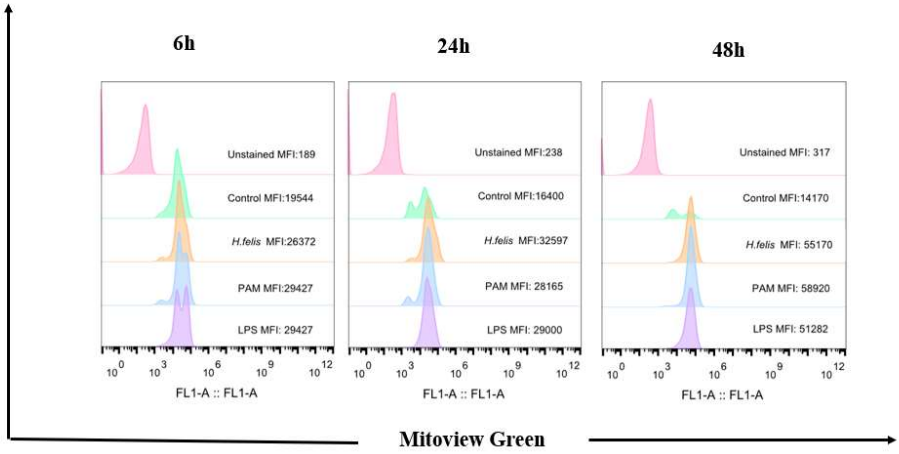
Figure 3.2: IL-10 secretion levels of unstimulated and stimulated B cells. Representative bar graphs show IL-10 secretion levels from unstimulated and *H.felis*, PAM3CSK4, and LPS stimulated-B cells. Each value represents two replicates analyzed using unpaired two-tailed Student's t-test in GraphPad Prism. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.3 *H. felis*- activation Increased Mitochondrial Mass in B Cells

Previous studies show that LPS (TLR4 ligand) stimulated-B cells increased their mitochondrial mass during the 6h incubation period (Caro Maldonado, 2014). However, the mitochondrial mass of *H. felis* stimulated-B cells was unknown. Therefore, in this part of the study, the effects of *H.felis* on mitochondrial mass were investigated. For this purpose, *H. felis*, PAM3CSK4, and LPS stimulated B cells were cultured at 6h, 24h, and 48h time points and after incubation, these groups were stained with Mitoview Green and analyzed by flow cytometry.

Figure 3.3A shows a representative histogram analysis of Mitoview Green for both the unstimulated control group and different stimulation groups. At 6h, 24h, and 48h time points mitochondrial mass of *H. felis*, PAM3CSK4, and LPS-stimulated B cells significantly increased compared to unstimulated B cells (Figure 3.3B).

A.



B.

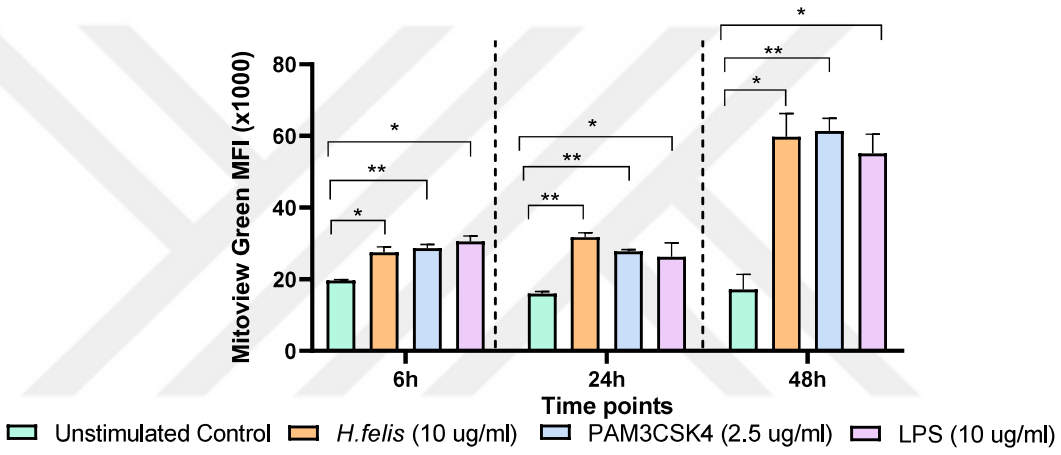


Figure 3.3: *H. felis*- activated B cells increased mitochondrial mass. (A) Representative histogram of mitochondrial mass (Mitoview Green) of *H.felis*, PAM3CSK4, and LPS-stimulated B cells after assessed by flow cytometry for 6, 24, and 48h. (B) Mitoview Green MFI values of the experiment are represented. Each value represents two replicates analyzed using unpaired two-tailed Student's t-test in GraphPad Prism. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.4 *H. felis* -activated B cells Increased Mitochondrial Membrane Potential.

B cells stimulated with LPS and IL-4 leads to two distinct populations in terms of mitochondrial mass and membrane potential. The first population increases both their mitochondrial mass and membrane potential and undergoes plasma class switch recombination. The second population increases their mitochondrial mass and membrane potential moderately and generates $CD138^{+}$ plasma cells (Jang et al, 2015). However, the mitochondrial membrane potential of *H. felis* stimulated-B cells has not been studied. In addition to mitochondrial mass, the mitochondrial membrane potential of *H. felis*, PAM3CSK4, and LPS-activated B cells was evaluated using Mitoview

633 and MitoStatus TMRE dyes at 6h, 24h, and 48h time points. As a negative control FCCP (25 µM), mitochondrial oxidative phosphorylation uncoupler, was used at 24h LPS group to disrupt the mitochondrial membrane potential.

Figure 3.4A shows a representative histogram analysis of Mitoview 633 and MitoStatus TMRE for both the unstimulated control group and various stimulation groups. At 6h, there were no noticeable changes in membrane potential. But at 24h and 48h time points mitochondrial membrane potential of *H. felis*, PAM3CSK4, and LPS stimulated B cells were increased significantly compared to unstimulated B cells (Figure 3.4B).

A.

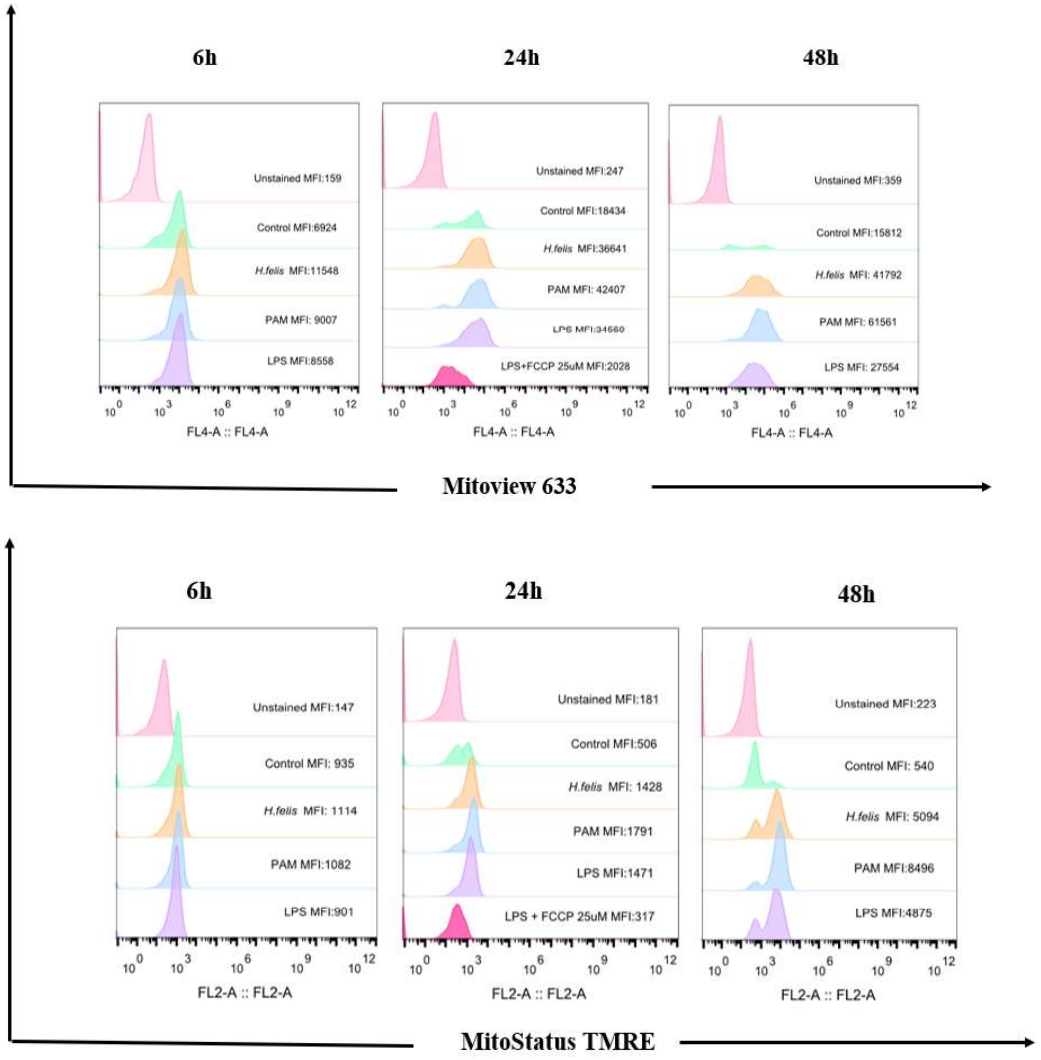


Figure 3.4: *H.felis*-activated B cells increased mitochondrial membrane potential.

B.

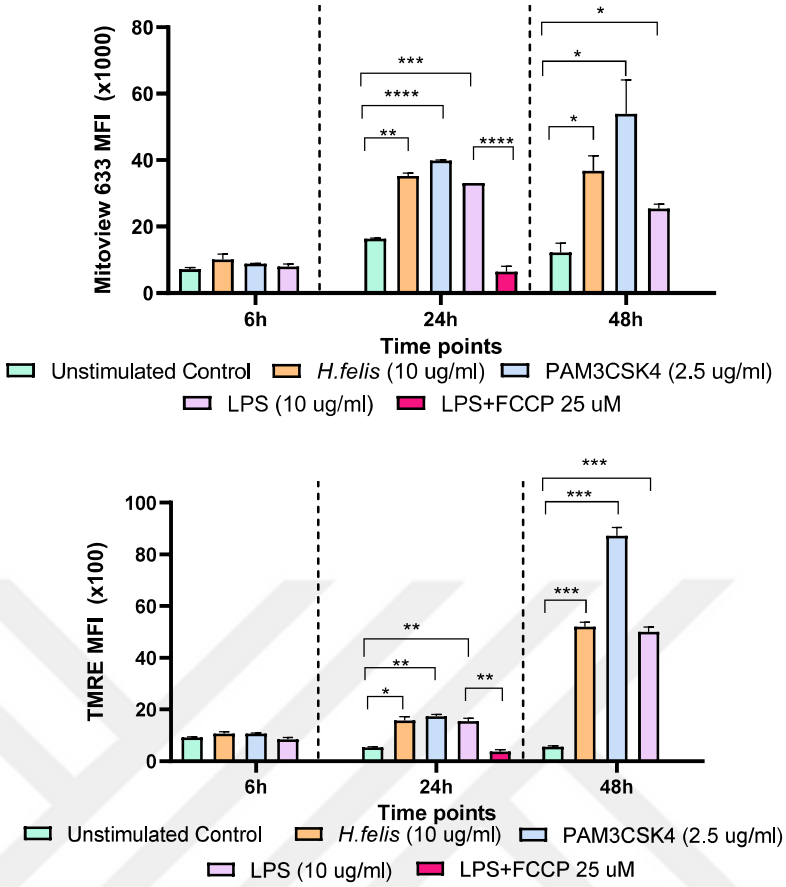


Figure 3.4 (continued): *H.felis*-activated B cells increased mitochondrial membrane potential. (A) Representative histogram of mitochondrial membrane potential (Mitoview 633 or MitoStatus TMRE) *H.felis*, PAM3CSK4, and LPS stimulated B cells assessed by flow cytometry for 6,24, and 48h. (B) Mitoview 633 or MitoStatus TMRE MFI values of the experiment are shown. Each value represents two replicates analyzed using unpaired two-tailed Student's t-test in GraphPad Prism. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.5 B cells which have high mitochondrial membrane potential also increased their IL-10 production.

To assess whether B cells with a high mitochondrial membrane potential secrete IL-10 or not; we isolated splenic B cells from IL-10 GFP reporter (VertX IL10 ^{egfp}) mice. The IL-10 GFP (VertX IL10 ^{egfp}) mice expresses an (IRES)- enhanced green fluorescent protein (e GFP) fusion protein downstream of the exon 5 of the IL-10 gene to provide IL-10 cytokine identification of IL-10^{+} myeloid and lymphoid cells. (Madan et al, 2009) In a previous study from our group has shown that a portion of *H. felis* stimulated B cells increased their IL-10 GFP signal detected in flow cytometry. (Sayı et al, 2011) In this study, we stimulated B cells with *H.felis*, PAM3CKS4, and LPS

for 6,24, and 48h and firstly assessed if these B cells increased their mitochondrial membrane potential or using Mitoview 633 flow staining. Figure 3.5A shows that all the stimulated groups enhanced mitochondrial membrane potential in IL-10 GFP reporter mice. Afterwards, at 6h, 24h and 48h we evaluated mitochondrial membrane potential of the IL-10 producing B cells with quadrant analysis. At 6h time there were no significant changes on IL-10^{+} Mitoview 633^{+} B cells. But at 24h and 48h time points; *H.felis*, PAM3CKS4, and LPS-stimulated B cells increased IL-10^{+} Mitoview 633^{+} B cells. (Figure 3.5B) These data show that in all stimulated B cell groups; high portion of the IL-10 producing B cells also have high mitochondrial potential. Lastly, we analyzed the IL-10 GFP signal of Mitoview 633^{+} B cells. According to our data, *H.felis*, PAM3CSK4, and LPS-stimulated Mitoview 633^{+} B cells increased their IL-10 GFP signal both at 24h and 48h time points compared to the unstimulated control group (Figure 3.5C).

A.

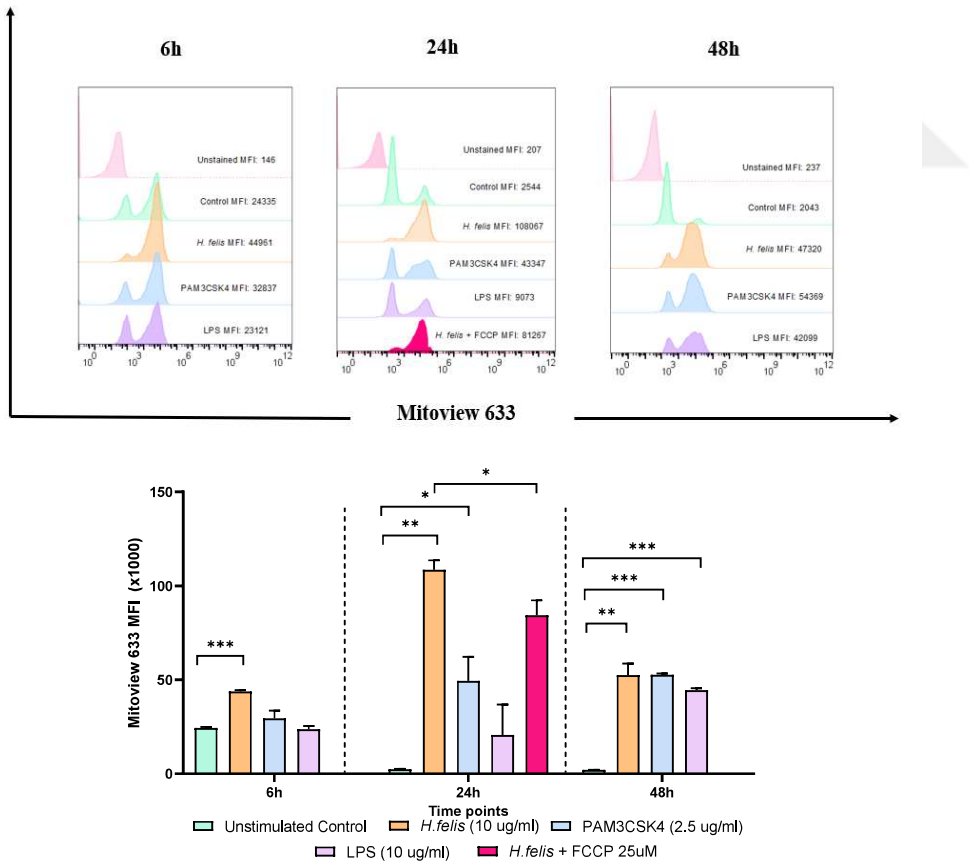


Figure 3.5: B cells with a high mitochondrial membrane potential also increased their IL-10 production.

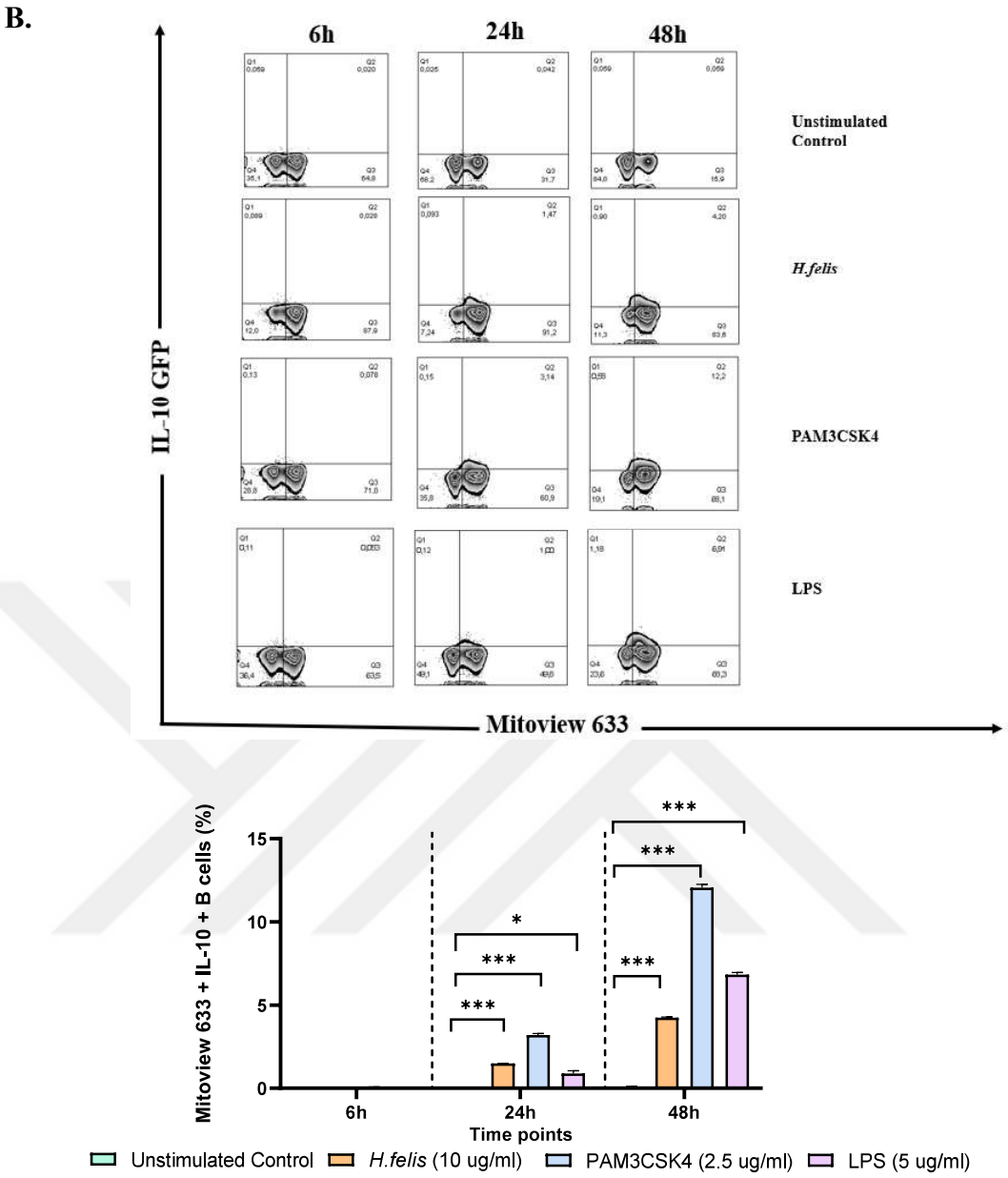


Figure 3.5 (continued): B cells with a high mitochondrial membrane potential also increased their IL-10 production.

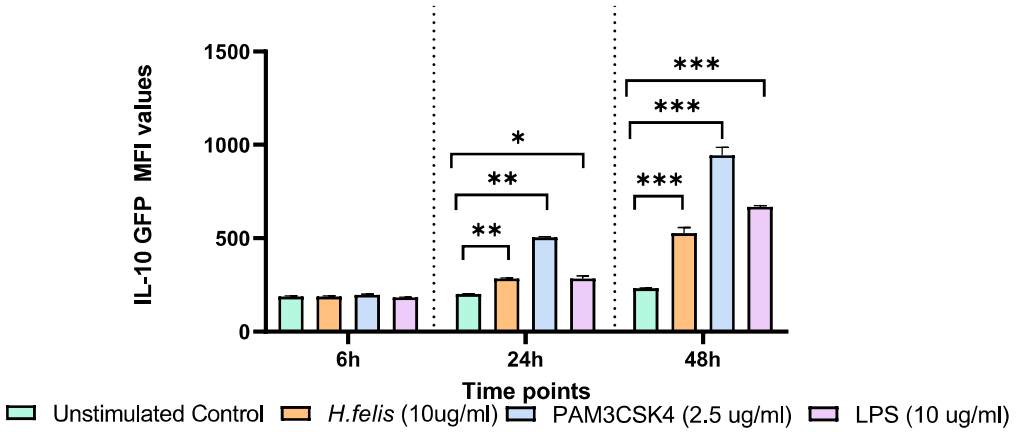


Figure 3.5 (continued): B cells with a high mitochondrial membrane potential also increased their IL-10 production. (A) Representative histogram and MFI values of mitochondrial membrane potential (Mitoview 633) of *H.felis*, PAM3CSK4, and LPS stimulated B cells assessed by flow cytometry at 6,24, and 48h. (B) Representative quadrant analysis and percentages of IL-10^{+}Mitoview 633^{+} B cells after assessed by flow cytometry for 6, 24, and 48h. (C)Representative histogram and MFI values of IL-10 GFP signal of Mitoview 633^{+} B cells after assessed by flow cytometry for 6, 24, and 48h. Each value represents two replicates analyzed using unpaired two-tailed Student's t-test in GraphPad Prism. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.6 mtDNA/nDNA Ratio is Decreased in B Cells stimulated with *H.felis*, PAM3CSK4, and LPS

mtDNA copy numbers, the elevated mtDNA:nDNA ratio and the level of mitochondrial gene expression are the main mitochondrial biogenesis markers (Popov et al,2020). While CpG-stimulated or CpG-anti-IgM stimulated B cells increased the mtDNA:nDNA ratio (Akkaya et al,2018); CD40L-IL-4 stimulated B cells decreased the mtDNA:nDNA ratio compared to the unstimulated group at 24h (Waters et al,2018). However, the mtDNA:nDNA ratio of *H. felis* stimulated-B cells has not been evaluated before. For this reason;i by Q-PCR experiment was performed to target amplification of cytochrome c oxidase subunit I (Cox1) (for the mitochondrial genome) and the 18S ribosomal subunit gene (Rps18) (for the nuclear genome). For almost every time point, stimulated B cells decreased their COX1:RPS18 ratio compared to the unstimulated control group, except for the LPS-treated B cell group at 24 h. (Figure 3.6).

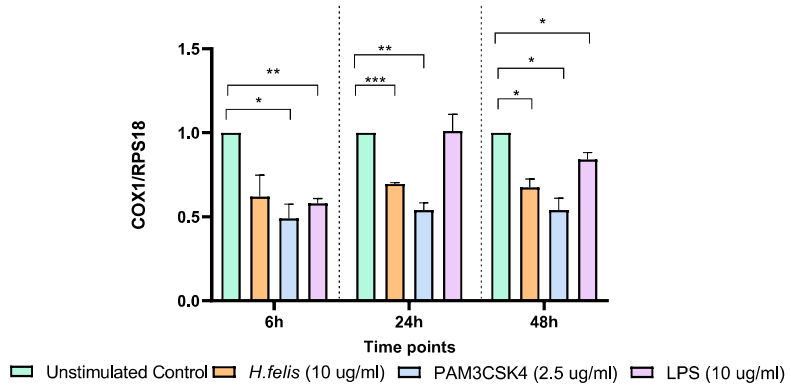


Figure 3.6: mtDNA:nDNA ratio is decreased with stimulation of B cells with *H.felis*, PAM3CSK4, and LPS. qPCR analysis of COX1:RPS18. Cell pellets from control and treated groups were collected and analyzed by qPCR. The graph was prepared using Graphpad Prism 8. Each value represents two replicates analyzed using Graphpad Prism's unpaired two-tailed Student's t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.7 Tfam mRNA level is increased with stimulation of B cells with *H.felis*, PAM3CSK4, and LPS

Tfam is one of the main mitochondrial transcription factor and marker of mitochondrial biogenesis. Studies reveal that Tfam is required for tissue-resident regulatory T cell maintenance and function (Fu et al,2018; Debnath et al,2022).Tfam expression is also induced in CpG-stimulated or CpG-anti- IgM stimulated B cells (Akkaya et al, 2018). However, the expression level of the Tfam gene in *H. felis* stimulated-B cells was unknown. To detect Tfam mRNA levels; RT-PCR was done by normalizing Tfam gene expression to beta 2-microglobulin housekeeping expression. As a result, in almost every time point, Tfam expression level was increased in all stimulated B cell groups.

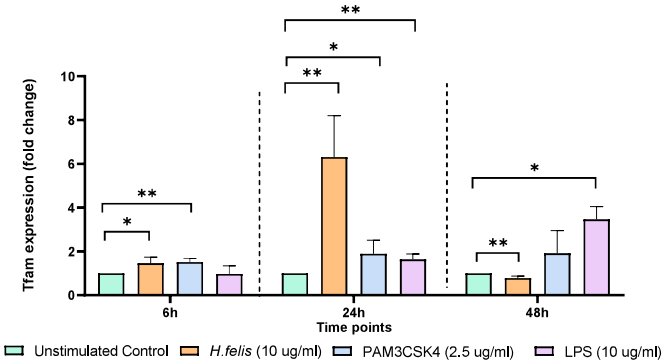


Figure 3.7: Tfam mRNA level is increased with stimulation of B cells with *H.felis*, PAM3CSK4, and LPS. RT-PCR analysis of Tfam gene. Cell pellets from control and treated groups were collected and analyzed by RT-PCR. The graph was prepared using Graphpad Prism 8. Each value represents two replicates analyzed using Graphpad Prism's unpaired two-tailed Student's t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4. DISCUSSION

B cells are adaptive immune cells that produce antibodies and secrete cytokines to mediate humoral immune responses. In addition to these effector B cells; in previous years, B cells that exert negative immunomodulatory effects were identified and called regulatory B cells (Abebe et al., 2021). Bregs produce IL-10, TGF-beta, and IL-35 for suppressing the function of other immune cells for maintaining tolerance and immune homeostasis (Dai et al., 2017).

Helicobacter pylori is a gram-negative, microaerophilic, and spiral-shaped bacterium and can lead to several gastric pathologies. More than 80% of *Helicobacter*-infected individuals do not show any symptoms and it can be ascribed to the suppressive properties of regulatory immune cells which increased against bacteria (Alexander et al., 2021).

IL-10 is an anti-inflammatory cytokine that exhibits immune regulatory functions. Sayi et al. have shown that B cells stimulated with *H. felis* antigen differentiate into IL-10-producing Bregs and these cells can induce differentiation of $CD4^+$ T cells to IL-10-producing regulatory Tr1 cells by direct B and T cell interactions for suppressing *Helicobacter*-associated pathologies. In addition to that, LPS-stimulated B cells also secrete IL-10 in a p38-dependent manner (Mion et al., 2014).

Metabolism is the sum of all anabolic and catabolic reactions which are the generation and breakdown of cellular substances respectively. In eukaryotic cells, glycolysis, oxidative phosphorylation, fatty acid oxidation, the pentose phosphate pathway, fatty acid synthesis, and amino acid synthesis are the major metabolic pathways. These pathways can influence B cell development, activation, differentiation, and function (O'Neill et al., 2016).

Oxidative phosphorylation comprises both the TCA cycle and ETC reactions to produce ATP. Immune responses can be activated by signals which are produced by metabolic intermediates and ATP. Hence, mitochondria are the prominent energy-producing organelle it plays important roles in maintaining and providing proper

immune responses (Angajala et al., 2018). Studies have shown that mitochondrial machinery components such as mitochondrial DNA (mtDNA), mitochondrial reactive oxygen species (mtROS), Tfam, ATP, cardiolipins, MAVS, and other mitochondrial proteins induce immune responses in macrophages, T cells, eosinophils, and dendritic cells (DCs) (Iwasaki et al., 2020). Recent studies focus on investigating the glycolytic, mitochondrial, and lipid metabolism of B cells. However, the immunometabolism of *Helicobacter*-infected B cells has not been elucidated yet.

This study mainly investigates the mitochondrial metabolism of *H. felis*-activated B cells. Firstly, we isolated naive B cells from the spleens of C57BL6 mice and then stimulated these B cells with TLR2 ligand *H. felis* for 6, 24, and 48h. Another TLR2 ligand PAM3CSK4 and TLR4 ligand LPS were used as control groups of the experiment at respective time points. Then we examined IL-10 production of *H.felis*, PAM3CSK4, and LPS-stimulated B cells by IL-10 ELISA. Comparable to the studies of Sayi et al. or Mion et al.; *H. felis*, PAM3CSK4, and LPS-stimulated B cells increased their IL-10 production indicating the suppressive capacity of that cells.

Immune cells which have functional mitochondria increase both mitochondrial mass and mitochondrial membrane potential (de Brito Monteiro et al., 2020). In this study, mitochondrial mass and membrane potential were analyzed by flow cytometry. Compared to the unstimulated control group, both the mass and membrane potential of mitochondria of all of the stimulant groups is increased at 24 and 48h time points (Figures 3.3 and 3.4) In literature, LPS-stimulated B cells enhanced mitochondrial mass at 6h time points. (Caro-Maldonado et al., 2014) Also in our study, *H. felis* and PAM3CSK4 stimulated B cell increased mitochondrial mass at 6h but at that time point membrane potential was not changed noticeably. These results indicate that B cells have functional mitochondria at later time points.

Ip et al showed that anti-inflammatory effect of IL-10 mediated by mitochondrial reprogramming of macrophages by regulating mTOR. And we wonder and asked whether B cells that have high mitochondrial membrane potential (Figure 3.4) also produce IL-10 or not. We isolated splenic B cells from IL-10 GFP reporter (VertX IL10 ^{egfp}) mice. The IL-10 GFP (VertX IL10 ^{egfp}) strain expresses an (IRES)- enhanced green fluorescent protein (eGFP) fusion protein downstream of the exon 5 of the IL-10 gene to provide IL-10 cytokine identification of IL-10^{+} myeloid and lymphoid cells. (Madan et al., 2009) After stimulation of these B cells with *H.felis*, PAM3CKS4, and

LPS for 6, 24, and 48h we analyzed their mitochondrial membrane potential profile. All of the stimulated groups enhanced mitochondrial membrane potential in IL-10 GFP reporter mice at 24 and 48h. Then we also analyzed the IL-10 GFP signal of Mitoview 633^+ B cells and percentages of Mitoview 633^+ IL-10 GFP^+ B cells. According to our data, *H.felis*, PAM3CSK4, and LPS-stimulated Mitoview 633^+ B cells increased their IL-10 GFP signal and percentages of Mitoview 633^+ IL-10 GFP^+ B cells both at 24h and 48h time points compared to the unstimulated control group (Figure 3.5B and Figure 3.5C). These data suggested that IL-10-producing B cells seem to use their mitochondria at 24h and 48h time points. But we also need to investigate IL-10 production of mitochondrial mass-positive B cells in our further studies.

Mitochondrial biogenesis involves mtDNA copy number, increased mtDNA:nDNA ratio, and elevated mitochondrial transcripts & proteins (Popov et al, 2020). One of the master mitochondrial regulators is the mitochondrial transcription factor A (Tfam). CpG-stimulated or CpG-anti-IgM stimulated B cells increased the mtDNA:nDNA ratio (Akkaya et al., 2018). In contrast, CD40L-IL-4 stimulated B cells decreased their mtDNA:nDNA ratio compared to the unstimulated control group at 24h (Waters et al., 2018).In our study mtDNA:nDNA ratio was evaluated by Q-PCR at 6, 24, and 48h time points. Our results were comparable to Waters et al's studies. *H. felis*, PAM3CSK4, and LPS-stimulated B cells decreased their mtDNA:nDNA ratio compared to the control group. Waters et al. suggested that activated OXPHOS can be a result of elevated mitochondrial transcripts instead of an increased mitochondrial DNA number. To check the dual role (mitochondrial transcription factor and mitochondrial biogenesis marker) of Tfam we then analyzed its expression level by using RT-QPCR. Tfam expression increased in *H. felis*, PAM3CSK4, and LPS-stimulated B cells compared to the control group indicating that mitochondrial biogenesis occurs in these groups independently of increasing mtDNA copy number.

5. CONCLUSION AND FUTURE PERSPECTIVES

Our study suggests that *H. felis*-activated B cells have active mitochondria, and increased oxidative phosphorylation for energy production and their IL-10 production can be related to their mitochondrial metabolism. The proposed model of the effect of *H.felis*-activated B cell mitochondrial metabolism can be seen in Figure 5.1. In this study; mitochondrial mass and membrane potential were evaluated. In future, other mitochondrial components such as mitochondrial ROS can be investigated in *Helicobacter*-stimulated B cells. Also, further studies should focus on the impact of the mitochondrial metabolism of *H.pylori*-infected pediatric and adult patient's blood and tissue samples to understand the mitochondrial metabolism of human samples instead of mice models.

Recent studies have shown that Tfam play crucial roles in differentiation and function of effector and regulatory T cells (Fu et al,2018; Debnath et al,2022). Because in our study Tfam expression was increased in stimulated B cells; using Tfam knockout mice, Tfam role in Breg differentiation and function can be investigated. Lastly, using single cell metabolism assays such as SCENITH, mitochondrial metabolism of immune cells can be evaluated for a better understanding of mitochondrial adaptations to activation and proper immune response.

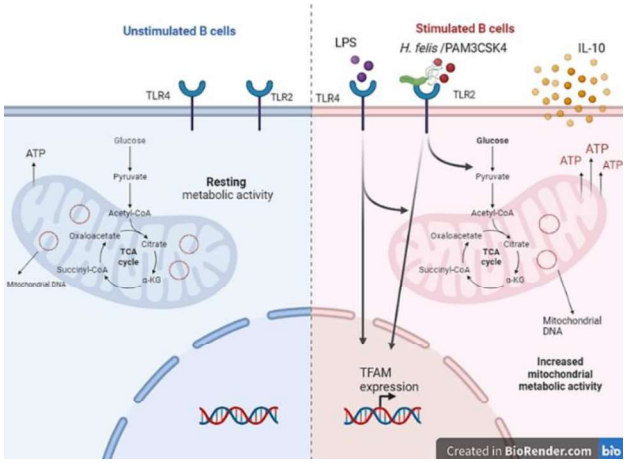


Figure 5.1: Proposal model of the effect of *H. felis* antigen, PAM3CSK4, and LPS on mitochondrial metabolism of B cells (Illustrated in Bio Render)

REFERENCES

Abebe, E. C., Dejenie, T. A., Ayele, T. M., Baye, N. D., Teshome, A. A., & Muche, Z. T. (2021). The role of regulatory B cells in health and diseases: A systemic review. In *Journal of Inflammation Research* (Vol. 14, pp. 75–84). Dove Medical Press Ltd. https://doi.org/10.2147/JIR.S286426

Ahmad, M., Wolberg, A., Chadi, ;, & Kahwaji, I. (n.d.). *Biochemistry, Electron Transport Chain*. https://www.ncb.nlm.nh.gov/books/NBK526105/#:~:text=The

Akira, S., & Takeda, K. (2004). Toll-like receptor signalling. In *Nature Reviews Immunology* (Vol. 4, Issue 7, pp. 499–511). Nature Publishing Group. https://doi.org/10.1038/nri1391

Akkaya, M., Traba, J., Roesler, A. S., Miozzo, P., Akkaya, B., Theall, B. P., Sohn, H., Pena, M., Smelkinson, M., Kabat, J., Dahlstrom, E., Dorward, D. W., Skinner, J., Sack, M. N., & Pierce, S. K. (2018). Second signals rescue B cells from activation-induced mitochondrial dysfunction and death. *Nature Immunology*, *19*(8), 871–884. https://doi.org/10.1038/s41590-018-0156-5

Alexander, S. M., Retnakumar, R. J., Chouhan, D., Devi, T. N. B., Dharmaseelan, S., Devadas, K., Thapa, N., Tamang, J. P., Lamtha, S. C., & Chattopadhyay, S. (2021). Helicobacter pylori in Human Stomach: The Inconsistencies in Clinical Outcomes and the Probable Causes. In *Frontiers in Microbiology* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2021.713955

Althuwaiqeb, S. A., & Bordoni, B. (n.d.). *Histology, B Cell Lymphocyte*. https://www.ncb.nlm.nh.gov/books/NBK560905/

Angajala, A., Lim, S., Phillips, J. B., Kim, J. H., Yates, C., You, Z., & Tan, M. (2018). Diverse roles of mitochondria in immune responses: Novel insights into immuno-metabolism. In *Frontiers in Immunology* (Vol. 9, Issue JUL). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2018.01605

Bibby, J. A., Purvis, H. A., Hayday, T., Chandra, A., Okkenhaug, K., Rosenzweig, S., Aksentijevich, I., Wood, M., Lachmann, H. J., Kemper, C., Cope, A. P., & Perucha, E. (2020). Cholesterol metabolism drives regulatory B cell IL-10 through provision of geranylgeranyl pyrophosphate. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-17179-4

Botos, I., Segal, D. M., & Davies, D. R. (2011). The structural biology of Toll-like receptors. In *Structure* (Vol. 19, Issue 4, pp. 447–459). https://doi.org/10.1016/j.str.2011.02.004

Breda, C. N. de S., Davanzo, G. G., Basso, P. J., Saraiva Câmara, N. O., & Moraes-Vieira, P. M. M. (2019). Mitochondria as central hub of the immune system. In *Redox Biology* (Vol. 26). Elsevier B.V. https://doi.org/10.1016/j.redox.2019.101255

Cai, X., Carlson, J., Stoicov, C., Li, H., Wang, T. C., & Houghton, J. (2005). Helicobacter felis eradication restores normal architecture and inhibits gastric cancer progression in C57BL/6 mice. *Gastroenterology*, *128*(7), 1937–1952. https://doi.org/10.1053/j.gastro.2005.02.066

Caro-Maldonado, A., Wang, R., Nichols, A. G., Kuraoka, M., Milasta, S., Sun, L. D., Gavin, A. L., Abel, E. D., Kelsoe, G., Green, D. R., & Rathmell, J. C. (2014). Metabolic Reprogramming Is Required for Antibody Production That Is Suppressed in Anergic but Exaggerated in Chronically BAFF-Exposed B Cells. *The Journal of Immunology*, *192*(8), 3626–3636. https://doi.org/10.4049/jimmunol.1302062

Chaudhry, R., & Varacallo, M. (n.d.). *Biochemistry, Glycolysis*. https://www.ncb.nlm.nih.gov/books/NBK482303/

Chi, H. (2022). Immunometabolism at the intersection of metabolic signaling, cell fate, and systems immunology. In *Cellular and Molecular Immunology* (Vol. 19, Issue 3, pp. 299–302). Springer Nature. https://doi.org/10.1038/s41423-022-00840-x

Dai, Y. C., Zhong, J., & Xu, J. F. (2017). Regulatory B cells in infectious disease (Review). In *Molecular Medicine Reports* (Vol. 16, Issue 1, pp. 3–10). Spandidos Publications. https://doi.org/10.3892/mmr.2017.6605

de Brito Monteiro, L., Davanzo, G. G., de Aguiar, C. F., Corrêa da Silva, F., Andrade, J. R. de, Campos Codo, A., Silva Pereira, J. A. da, Freitas, L. P. de, & Moraes-Vieira, P. M. (2020). M-CSF- and L929-derived macrophages present distinct metabolic profiles with similar inflammatory outcomes. *Immunobiology*, *225*(3). https://doi.org/10.1016/j.imbio.2020.151935

El-Zayat, S. R., Sibaii, H., & Mannaa, F. A. (2019). Toll-like receptors activation, signaling, and targeting: an overview. *Bulletin of the National Research Centre*, *43*(1). https://doi.org/10.1186/s42269-019-0227-2

Fillatreau, S., Sweenie, C. H., McGeachy, M. J., Gray, D., & Anderton, S. M. (2002). B cells regulate autoimmunity by provision of IL-10. *Nature Immunology*, *3*(10), 944–950. https://doi.org/10.1038/ni833

Fox, J. G. (2002). The non-H pylori helicobacters: Their expanding role in gastrointestinal and systemic diseases. In *Gut* (Vol. 50, Issue 2, pp. 273–283). BMJ Publishing Group. https://doi.org/10.1136/gut.50.2.273

Fu, Y., Wang, L., Yu, B., Xu, D., & Chu, Y. (2022). Immunometabolism shapes B cell fate and functions. In *Immunology* (Vol. 166, Issue 4, pp. 444–457). John Wiley and Sons Inc. https://doi.org/10.1111/imm.13499

Ganeshan, K., & Chawla, A. (2014). Metabolic regulation of immune responses. In *Annual Review of Immunology* (Vol. 32, pp. 609–634). Annual Reviews Inc. https://doi.org/10.1146/annurev-immunol-032713-120236

Guevara, B., & Cogdill, A. G. (2020). Helicobacter pylori: A Review of Current Diagnostic and Management Strategies. In *Digestive Diseases and Sciences* (Vol. 65, Issue 7, pp. 1917–1931). Springer. https://doi.org/10.1007/s10620-020-06193-7

Heine, G., Niesner, U., Chang, H. D., Steinmeyer, A., Zügel, U., Zuberbier, T., Radbruch, A., & Worm, M. (2008). 1,25-dihydroxyvitamin D3 promotes IL-10 production in human B cells. *European Journal of Immunology*, *38*(8), 2210–2218. https://doi.org/10.1002/eji.200838216

***HongzhiiSun Xiangdongg Wang Editors Mitochondrial DNA and Diseases*.** (n.d.). http://www.springer.com/series/5584

Hosomi, K., & Kunisawa, J. (2020). Diversity of energy metabolism in immune responses regulated by micro-organisms and dietary nutrition. In *International Immunology* (Vol. 32, Issue 7, pp. 447–454). Oxford University Press. https://doi.org/10.1093/intimm/dxaa020

Ip, W. K. E., Hoshi, N., Shouval, D. S., Snapper, S., & Medzhitov, R. (2017). Anti-inflammatory effect of IL-10 mediated by metabolic reprogramming of macrophages. *Science*, *356*(6337), 513–519. https://doi.org/10.1126/science.aal3535

Iwasaki, Y., Takeshima, Y., & Fujio, K. (2020). Basic mechanism of immune system activation by mitochondria. In *Immunological Medicine* (Vol. 43, Issue 4, pp. 142–147). Taylor and Francis Ltd. https://doi.org/10.1080/25785826.2020.1756609

Jang, K. J., Mano, H., Aoki, K., Hayashi, T., Muto, A., Nambu, Y., Takahashi, K., Itoh, K., Taketani, S., Nutt, S. L., Igarashi, K., Shimizu, A., & Sugai, M. (2015). Mitochondrial function provides instructive signals for activation-induced B-cell fates. *Nature Communications*, *6*. https://doi.org/10.1038/ncomms7750

Jansen, K., Cevhertas, L., Ma, S., Satitsuksanoa, P., Akdis, M., & van de Veen, W. (2021). Regulatory B cells, A to Z. In *Allergy: European Journal of Allergy and Clinical Immunology* (Vol. 76, Issue 9, pp. 2699–2715). John Wiley and Sons Inc. https://doi.org/10.1111/all.14763

Junge, W., & Nelson, N. (2015). ATP synthase. In *Annual Review of Biochemistry* (Vol. 84, pp. 631–657). Annual Reviews Inc. https://doi.org/10.1146/annurev-biochem-060614-034124

Kawasaki, T., & Kawai, T. (2014). Toll-like receptor signaling pathways. In *Frontiers in Immunology* (Vol. 5, Issue SEP). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2014.00461

Lam, W. Y., Becker, A. M., Kennerly, K. M., Wong, R., Curtis, J. D., Llufrio, E. M., McCommis, K. S., Fahrmann, J., Pizzato, H. A., Nunley, R. M., Lee, J., Wolfgang, M. J., Patti, G. J., Finck, B. N., Pearce, E. L., & Bhattacharya, D. (2016). Mitochondrial Pyruvate Import Promotes Long-Term Survival of Antibody-Secreting Plasma Cells. *Immunity*, *45*(1), 60–73. https://doi.org/10.1016/j.immuni.2016.06.011

Madan, R., Demircik, F., Surianarayanan, S., Allen, J. L., Divanovic, S., Trompette, A., Yogev, N., Gu, Y., Khodoun, M., Hildeman, D.,

Boespflug, N., Fogolin, M. B., Gröbe, L., Greweling, M., Finkelman, F. D., Cardin, R., Mohrs, M., Müller, W., Waisman, A., … Karp, C. L. (2009). Nonredundant Roles for B Cell-Derived IL-10 in Immune Counter-Regulation. *The Journal of Immunology*, *183*(4), 2312–2320. https://doi.org/10.4049/jimmunol.0900185

Makowski, L., Chaib, M., & Rathmell, J. C. (2020). Immunometabolism: From basic mechanisms to translation. In *Immunological Reviews* (Vol. 295, Issue 1, pp. 5–14). Blackwell Publishing Ltd. https://doi.org/10.1111/imr.12858

Marshall, J. S., Warrington, R., Watson, W., & Kim, H. L. (2018). An introduction to immunology and immunopathology. In *Allergy, Asthma and Clinical Immunology* (Vol. 14). BioMed Central Ltd. https://doi.org/10.1186/s13223-018-0278-1

Martínez-Reyes, I., & Chandel, N. S. (2020). Mitochondrial TCA cycle metabolites control physiology and disease. In *Nature Communications* (Vol. 11, Issue 1). Nature Research. https://doi.org/10.1038/s41467-019-13668-3

Mauri, C., & Bosma, A. (2012). Immune regulatory function of B cells. In *Annual Review of Immunology* (Vol. 30, pp. 221–241). https://doi.org/10.1146/annurev-immunol-020711-074934

Mauri, C., Gray, D., Mushtaq, N., & Londei, M. (2003). Prevention of arthritis by interleukin 10-producing B cells. *Journal of Experimental Medicine*, *197*(4), 489–501. https://doi.org/10.1084/jem.20021293

Medzhitov, R., Preston-Hurlburt, P., & Janeway, C. A. (1997). *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity*.

Mégraud, F., Bessède, E., & Varon, C. (2015). Helicobacter pylori infection and gastric carcinoma. In *Clinical Microbiology and Infection* (Vol. 21, Issue 11, pp. 984–990). Elsevier. https://doi.org/10.1016/j.cmi.2015.06.004

Mion, F., Tonon, S., Toffoletto, B., Cesselli, D., Pucillo, C. E., & Vitale, G. (2014). IL-10 production by B cells is differentially regulated by immune-mediated and infectious stimuli and requires p38 activation. In *Molecular Immunology* (Vol. 62, Issue 2, pp. 266–276). Elsevier Ltd. https://doi.org/10.1016/j.molimm.2014.05.018

Mizoguchi et al_2002. (n.d.).

Murphy, K. (Kenneth M.), Weaver, C., & Janeway, C. (n.d.). *Janeway's immunobiology*.

Okuno, D., Iino, R., & Noji, H. (2011). Rotation and structure of FoF1-ATP synthase. In *Journal of Biochemistry* (Vol. 149, Issue 6, pp. 655–664). https://doi.org/10.1093/jb/mvr049

O'Neill, L. A. J., Kishton, R. J., & Rathmell, J. (2016). A guide to immunometabolism for immunologists. In *Nature Reviews Immunology* (Vol. 16, Issue 9, pp. 553–565). Nature Publishing Group. https://doi.org/10.1038/nri.2016.70

Popov, L. D. (2020). Mitochondrial biogenesis: An update. In *Journal of Cellular and Molecular Medicine* (Vol. 24, Issue 9, pp. 4892–4899). Blackwell Publishing Inc. https://doi.org/10.1111/jcmm.15194

Rosser, E. C., & Mauri, C. (2015). Regulatory B Cells: Origin, Phenotype, and Function. In *Immunity* (Vol. 42, Issue 4, pp. 607–612). Cell Press. https://doi.org/10.1016/j.immuni.2015.04.005

Sayi, A., Kohler, E., Toller, I. M., Flavell, R. A., Müller, W., Roers, A., & Müller, A. (2011). TLR-2–Activated B Cells Suppress Helicobacter -Induced Preneoplastic Gastric Immunopathology by Inducing T Regulatory-1 Cells . *The Journal of Immunology*, *186*(2), 878–890. https://doi.org/10.4049/jimmunol.1002269

Simpson, K. W., Strauss-Ayali, D., Scanziani, E., Straubinger, R. K., Mcdonough, P. L., Straubinger, A. F., Chang, Y.-F., Domeneghini, C., Arebi, N., & Calam, J. (2000). Helicobacter felis Infection Is Associated with Lymphoid Follicular Hyperplasia and Mild Gastritis but Normal Gastric Secretory Function in Cats. In *INFECTION AND IMMUNITY* (Vol. 68, Issue 2). https://journals.asm.org/journal/iai

Talley, J. T., & Mohiuddin, S. S. (n.d.). *Biochemistry, Fatty Acid Oxidation*. https://www.ncb.nlm.nh.gov/books/NBK556002/

Wang, Y., Song, E., Bai, B., & Vanhoutte, P. M. (2016). Toll-like receptors mediating vascular malfunction: Lessons from receptor subtypes. In *Pharmacology and Therapeutics* (Vol. 158, pp. 91–100). Elsevier Inc. https://doi.org/10.1016/j.pharmthera.2015.12.005

Waters, L. R., Ahsan, F. M., Wolf, D. M., Shirihai, O., & Teitell, M. A. (2018). Initial B Cell Activation Induces Metabolic Reprogramming and Mitochondrial Remodeling. *IScience*, *5*, 99–109. https://doi.org/10.1016/j.isci.2018.07.005

Wolf, S. D., Dittel, B. N., Hardardottir, F., & Janeway, C. A. (n.d.). Experimental Autoimmune Encephalomyelitis Induction in Genetically B Cell-deficient Mice. In *J. Exp. Med*. http://rupress.org/jem/article-pdf/184/6/2271/1109799/5340.pdf

Zorova, L. D., Popkov, V. A., Plotnikov, E. Y., Silachev, D. N., Pevzner, I. B., Jankauskas, S. S., Babenko, V. A., Zorov, S. D., Balakireva, A. v., Juhaszova, M., Sollott, S. J., & Zorov, D. B. (2018). Mitochondrial membrane potential. *Analytical Biochemistry*, *552*, 50–59. https://doi.org/10.1016/j.ab.2017.07.009

CURRICULUM VITAE

Name Surname : Zeynep Nur Şentürk

EDUCATION :

B.Sc. : Yıldız Technical University (2015 – 2019), Faculty of Art & Science, Bachelor of Science in Molecular Biology and Genetics, High Honour Student (GPA: 3.84 / 4.00) / İstanbul / Turkey**.** .

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Senturk Z.N.**, Calci M., Deniz B., Sayi Yazgan A., (2022): *Helicobacter* activated B cells increased their mitochondrial metabolism. The 8th International Congress of the Molecular Biology Association of Turkey, June 09-12, 2022, Istanbul/TURKEY. (Poster Presentation)
- **Senturk Z.N,** Deniz B., Akdag I., Calci M., Ozdemir N., Sayi Yazgan A., (2022): Investigation of mitochondrial metabolism of *Helicobacter*-activated B cells. The 5th International Molecular Immunology and Immunogenetics Congress, October 20-22, 2022, İzmir/TURKEY. (Poster Presentation)

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

- Calci M., Akdag I., **Senturk Z.N,** Deniz B., Sayi Yazgan A., (2021): Effect of B-cell activating factor on *Helicobacter*-activated B cell survival and differentiation. 6th European Congress of Immunology, Online (Poster Presentation)
- Calci M., Deniz B., **Senturk Z.N.**, Sayi Yazgan A., (2022): B Cells Undergo Metabolic Reprogramming upon *Helicobacter* Activation. The 8th International Congress of the Molecular Biology Association of Turkey, June 09-12, 2022, Istanbul/TURKEY. (Poster Presentation)
- Deniz B., Calci M., **Senturk Z.N.**, Sayi Yazgan A., (2022): Glycolysis Inhibitor 2-DG Suppresses Proliferation, Survival and IL-10 Secretion of *Helicobacter* Activated B Cells. The 8th International Congress of the Molecular Biology Association of Turkey, June 09-12, 2022, Istanbul/TURKEY. (Poster Presentation)
- Deniz B., **Senturk Z.N,** Calci M., Akdag I., Ozdemir N., Tanil M., Abdul C.B., Sayi Yazgan A., (2022): The effect of glycolysis and OXPHOS inhibition on *Helicobacter* activated B cell's. The 5th International Molecular Immunology and Immunogenetics Congress, October 20-22, 2022, İzmir/TURKEY. (Poster Presentation)

- Akdag I., **Senturk Z.N,** Calci M., Deniz B., Ozdemir N., Sayi Yazgan A., (2022): The effect of *Helicobacter*-mediated stimulation on glucose uptake, intracellular glucose transporters and glycolysis enzymes levels in B lymphocytes. The 5th International Molecular Immunology and Immunogenetics Congress, October 20-22, 2022, İzmir/TURKEY. (Poster Presentation)
- Ozdemir N.,Güller D., Usta M., Urgancı N., Akdag I., **Senturk Z.N,** Oktem Oktullu S., Sayi Yazgan A., (2022): *Helicobacter pylori* virulence factors and T-cell cytokines in *Helicobacter pylori*-infected pediatric patients with gastritis. The 5th International Molecular Immunology and Immunogenetics Congress, October 20-22, 2022, İzmir/TURKEY. (Poster Presentation)