REGULATING INFLAMMATION THROUGH EXERCISE-MEDIATED TRAINED IMMUNITY OR GENETIC MANIPULATION OF THE INTERFERON LOCUS

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A DISSERTATION SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN 2023

GRADUATE PROGRAM IN KINESIOLOGY AND HEALTH SCIENCE

YORK UNIVERSITY, TORONTO, ONTARIO

Abstract

Inflammation is a protective early immune response to infection or injury. A balanced inflammatory response, which is self-limiting and self-resolving, is essential to restore homeostasis. However, dysregulated inflammation is underlying many chronic diseases and infections. Strategies for balancing inflammation can be achieved in two ways 1) by inducing adaptations in immune cells through environmental/lifestyle factors such as exercise and 2) by genetically manipulating or pharmaceutically targeting key immune modulatory factors, such as interferons. In my thesis, I employed both approaches. First, we explored whether exercise training could induce adaptations in bone marrow-derived macrophages, resulting in trained immunity, known to occur in immune cells due to persistent metabolic and epigenetic changes. Data from this study suggest that chronic moderate exercise can influence the inflammatory responses of macrophages by reprogramming their metabolic and epigenetic landscape. Second, we evaluated the role of specific type I interferons (IFN-α and IFN-β), cytokines known for their antiviral and immunomodulatory functions, in virus infection and PAMPs (pathogen-associated molecular patterns) mediated inflammatory responses. To study this, we generated unique knockout (KO) mice of type I interferons (IFN-Is) locus and evaluated viral clearance and other parameters such as cytokine, interferon stimulated genes (ISG) signatures, and bone marrow hematopoiesis. Data from this study showed the importance of IFN-αs in early viral clearance and other parameters that measured antiviral and inflammatory responses, which encourages the need to study the individual IFN Is in isolation using reliable mouse models to uncover their specific roles and targets. Overall, data from both approaches contributed significantly to improving our understanding of the mechanisms behind exercise and IFN-I mediated regulation of inflammatory responses.

Acknowledgement

Four and half years of sincere effort to grow! First and foremost, I would like to thank my supervisor, Dr. Ali Abdul-Sater, for the opportunity to do a Ph.D. in his lab. I am forever grateful for the trust and the space you have given me to learn, grow, and rediscover myself in the ways that worked for me.

Next, I sincerely thank my committee members for all the support and guidance you provided me, which were always encouraging and thoughtful and helped me orient myself and grow.

A huge thanks to my talented and hardworking lab members. Ardavan, Bipen, Amamdeep, Fatemah, Safoura, Sunpreet, Yitian, Sahib, Alita, Ali A and Ali M. Thanks for inspiring me in many ways.

Two more people I would love to thank here are Shailee and Catherine. Shailee, thank you for your motivation when I wanted to return and do this Ph.D. and during this journey. Catherine, you are an amazing person. You have always answered my questions with no hesitation.

Finally, without the support and patience of my family, this journey would have been impossible.

I am eternally grateful to God for everything I learned through this journey!

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List of Abbreviations

A

AP-1- Activator protein 1
ALRs- AIM2 like receptors
ACLY - ATP citrate lyase
AMPK - AMP-activated protein kinase
Akt - Protein kinase B (PKB)
APCs - Antigen-presenting cells
ATF-2 - Activating transcription factor 2
ANOVA - Analysis of variance
ARG1 - Arginase-1
ATAC-seq - Assay of Transposase Accessible Chromatin sequencing

B

BMDM – Bone Marrow Derived Macrophages
BSA – Bovine Serum Albumin
BCAA - branched-chain amino acids
BMI – Body mass index
BST2 - Bone marrow stromal cell antigen 2

C

cGAMP – Cyclic Guanosine Monophosphate–Adenosine Monophosphate
cGAS – Cyclic Guanosine Monophosphate–Adenosine Monophosphate Synthase
CDN – Cyclic Dinucleotides
cDNA – Complementary Deoxyribonucleic Acid
CD-Cluster of differentiation
CLRs- C-type lectin receptors
CCL2 - chemokine ligand 2 **(MCP-1)**
CXCL10 - C-X-C motif chemokine ligand 10 (**IP-10**)
CX3CL1 - C-X3-C motif chemokine ligand 1 (**KC)**
CPT-1 - Carnitine palmitoyl transferase I
CIC - citrate carrier
CHIKV – Chikungunya virus
CTL – Cytotoxic lymphocytes
CML - Chronic myeloid leukemia
CART - Chimeric antigen receptor T cell Therapy
D
DC – Dendritic Cells
DMEM – Dulbecco's Modified Eagle Medium
DNA – Deoxyribonucleic Acid
dsDNA – Double-Stranded Deoxyribonucleic Acid
dsRNA – Double-Stranded Ribonucleic Acid
DTT – Dithiothreitol
DAMPs- Danger associated molecular patterns
DMEM - Dulbecco's Modified Eagle Medium
DESeq 2 - Differential gene expression analysis based on the negative binomial distribution
DEGs - Differentially expressed genes
E
EDTA– Ethylenediaminetetraacetic Acid
ER – Endoplasmic Reticulum
ETC – Electron transport chain

F
FBS – Fetal Bovine Serum
FAO – Fatty acid oxidation
G
GPPS – Glutamine, sodium Pyruvate (NaPy), Penicillin and Streptomycin
GLUT1 – Glucose transporter 1
GCMS - Gas chromatography–mass spectrometry
GM-CSF - Granulocyte-macrophage colony-stimulating factor
H
HCL – Hydrochloric Acid

Hif1-α - hypoxia-inducible factor-1alpha
HIV - Human immunodeficiency virus
HCV - hepatitis C virus
HBV - hepatitis B virus
HuIFN – Human IFN
HIF1a - Hypoxia-inducible factor-1α
HMOX-1 - Heme-oxygenase-1

I

IFN – Interferon
IκB – Inhibitor of Nuclear Factor Kappa-B
IKK – Inhibitory Kappa-B Kinase
IL-1 – Interleukin-1
IL-1β – Interleukin-1-Beta
IL-4 – Interleukin-4
IL-6 – Interleukin-6
IL-8 – Interleukin-8
IL-12 – Interleukin-12
IL-18 – Interleukin-18
iNOS – Inducible Nitric Oxide Synthase
IRAK – IL-1R-Associated Kinase
ISG- Interferon Stimulated Genes
ICAM-1 - intercellular adhesion molecule
IRF - Interferon regulatory factors
IFNAR - Interferon alpha receptors
IFNLR -Interferon lambda receptors
ISGF3 - IFN-stimulated gene factor-3
IRepGs - Interferon repressed genes
IRG1 - Immune-responsive gene 1
IFN-β^{only} – Interferon alpha knock out
IFN-βKO - Interferon beta knock out
IFNARKO – Interferon receptor knock out

J

JAKs – Janus Kinases

K

KO- Knock Out

L

LCM – L929 Conditioned Media

LPS – Lipopolysaccharides
LRR – Leucine-Rich Repeat-containing Proteins
LCMS/MS - Liquid Chromatography with tandem mass spectrometry
LCMV - Lymphocytic Choriomeningitis Virus
LCMV Cl-13 - Lymphocytic Choriomeningitis Virus Clone 13
LCMV Arm - Lymphocytic Choriomeningitis Virus Arm strong

M
MAPK – Mitogen-Activated Protein Kinase
MAVS – Mitochondrial Antiviral Signaling Protein
MDP – Bacterial Muramyl Dipeptide
M-MuLV – Moloney Murine Leukemia Virus
MyD88 – Myeloid Differentiation Primary Response Protein-88
MDA5 - melanoma differentiation-associated protein 5
MCP-1 - Monocyte chemoattractant protein-1
MTOR - mammalian target of rapamycin
MuIFN – Murine IFN
M1XA
MHC Major Histocompatibility complex
N
NaCl – Sodium Chloride
NEAA – Non-Essential Amino Acid
NF-κB – Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B-Cells
NK – Natural Killer Cells
NLR – NOD-like Receptors
NOD – Nucleotide-binding Oligomerization Domain
NP – Nonionic Polyoxyethylene
NZB - NZBWF1/J mice strain
nsp 13 Non-structural protein 13
nsp15 Non-structural protein 115
O
OXPHOS- oxidative phosphorylation
orf8 - viral accessory protein

P
PAMP – Pathogen-Associated Molecular Patterns
PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
Poly dA:dT – Poly (deoxyAdenylic-deoxyThymidylic)
Poly I:C – PolyInosinic–polyCytidylic
PRR – Pattern Recognition Receptors

PGI2 - prostaglandin I2
PI3K - Phosphoinositide 3-kinases
PPAR-γ - proliferator-activated receptor γ
PGC-1a - Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1alpha
PDL-1 - Programmed death-ligand 1
pDCs - Plasmacytoid dendritic cells
PCA - Principal component analysis
PFK2 - Phosphofructokinase
PKR - protein kinase R

R
RIG-I – Retinoic Acid-Inducible Gene I
RIP2 – Receptor Interacting Protein-2
RLR – RIG-I-Like Receptors
RNA – Ribonucleic Acid
RPM – Revolutions Per Minute
ROS – Reactive oxygen species
RPMI - Roswell Park Memorial Institute Medium
RANTES - Chemokine ligand 5 (CCL5)
S
SDS-PAGE – Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
STING – Stimulator of Interferon Genes
STATs - Signal transducer and activator of transcription
SOCS1 - Suppressor of cytokine signaling 1
T
TAK1 – TGF-β Activated Kinase
TBK1 – TANK-Binding Kinase-1
TBST – Tris-Buffered Saline and Tween
TGFβ – Transforming Growth Factor Beta
TIR – Toll-IL-1-Receptor
TLR – Toll-like Receptors
TNFα – Tumor Necrosis Factor-Alpha
TRAF6 – TNF Receptor-Associated Factor 6
TRIF -TIR domain- containing adaptor protein inducing IFN-β
TRAF – Tumor necrosis factor receptor associated factor
TBK1 - TANK-binding kinase 1
TCA cycle – Tricarboxylic acid cycle
TYK2 - Tyrosine kinase 2
TMM - Trimmed mean of M-values
TGFb - Transforming growth factor beta

TKO – Total knockout

U

URTI – Upper Respiratory Tract Infections

UHPLCMS - Ultrahigh-pressure (or performance) liquid chromatography (UHPLC) mass spectrometry

UPLC-qTOF-MS - ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry

USP18 - Ubiquitin Specific Peptidase 18

V

VCAM - vascular cell adhesion molecule

W

WT – Wild Type

1 Chapter 1: General introduction of the thesis

Inflammation is a protective early immune response to infection or injury.[1–4] It is a well-coordinated innate immune response that demands enormous metabolic energy and host damage during the process but resolves on its own to restore homeostasis. A dysregulated inflammation, on the other hand, will lead to multiple organ and tissue destruction and may also lead to the risk of sepsis.[5] Thus, inflammation dysregulation is underlying many chronic diseases and infections. Severe COVID-19 disease is also related to excessive dysregulated inflammation.[6–8]

Inflammation involves the secretion of various inflammatory mediators that orchestrate the coordinated response. Tissue-resident and recruited immune cells secrete these biomolecules, including cytokines, chemokines, enzymes, certain proteins, etc.[9] To balance the inflammation is to balance the production and the functions of these mediators. This can be achieved by training the immune system to produce a balanced inflammatory response through lifestyle factors such as exercise or by genetically manipulating or pharmaceutically targeting central inflammatory signalling pathways.

Exercise can modulate immune responses depending on its intensity, model and duration.[10–12] An in-depth understanding of the mechanisms behind the exercise-mediated adaptations is crucial to design exercise therapies or pharmaceutical mimics for patients with disease conditions. Physical activity alters body homeostasis by changing energy demand, O_2 availability, tissue stress or damage etc. These changes result in the release of various molecules into the circulation, including myokines, metabolites, hormones, cytokines, chemokines, specific peptides, nucleotides, etc. Collectively they are named “Exokines”, any biomolecules secreted into the circulation upon exercise.[9] Through circulation, these molecules can reach lymphoid organs such as the bone

marrow or thymus, where immune cells originate and/or differentiate. Thus, these changes can lead to adaptations in the immune cells residing in these lymphoid organs.

In this study, we explored whether moderate exercise could cause persistent adaptation in bone marrow-derived macrophages, resulting in trained immunity, known to occur in innate immune cells due to persistent metabolic and epigenetic changes following primary stimulations. We show that long-term moderate-intensity training of mice leads to metabolic rewiring and changes in chromatin accessibility, which, in turn, causes a reduction in the inflammatory responses of macrophages when stimulated with lipopolysaccharide (LPS). Overall, our data suggest that chronic moderate exercise can influence the inflammatory responses of macrophages by reprogramming their metabolic and epigenetic landscape.

Similarly, a clear understanding of the signalling components and their functions is necessary to target central immune signalling pathways that regulate inflammation. One such crucial signalling pathway is the type I interferon (IFN I) pathway, known for its antiviral functions. In addition, it has recently been implicated in various chronic diseases, including cancer, autoimmune diseases and chronic infections. Dysregulated IFN I response is also associated with the COVID-19 cytokine storm.[6–8,13,14]

IFN Is comprise over a dozen subtypes and are clustered as intronless multigene families.[15] This signalling pathway has received much appreciation due to its wide range of functions in infections and chronic disorders. However, a complete understanding of the functional differences between the subtypes is still lacking. In this study, we employed multiple knockout (KO) mice of IFN I locus to study functional differences between the main IFN I subtypes (IFN-α and IFN–β). The genetic manipulations were carried out to enable the subsequent re-introduction of individual IFN-

α subtypes to make knock-in mice which will serve as a reliable model to study the unique roles of IFN Is independently. Overall, this study showed the importance of IFN-αs in early viral clearance and showed altered cytokine, ISG signatures, and bone marrow hematopoiesis among genotypes.

1.1 Overall objective of the thesis

In this thesis, we employed two approaches to balance the inflammatory response by investigating (1) exercise mediated adaptations in macrophages, and (2) how the loss of IFN-α versus IFN-β alter immune responses. Our overarching objective was to understand the mechanisms behind exercise-mediated adaptations that reduced inflammation in Bone marrow derived macrophages (BMDMs) and IFN I mediated inflammatory signalling upon infection or Pathogen associated molecular patterns (PAMPs) induction. Findings from these approaches will contribute to designing precise therapies or pharmaceutical targets to treat dysregulated inflammation.

2 Chapter 2: Literature review

2.1 Overview of the immune system [16]

The immune system's primary function is to protect our body from foreign viruses, bacteria, fungi, or parasites. Our immune system consists of two arms: the innate and adaptive immune systems. Although each arm has different functional properties and responsibilities, they work in concert to preserve the body's homeostasis. The innate immune system acts fast and is the first line of defence, initiating an inflammatory response. The adaptive immune system depends on innate immune components for its initiation; therefore, it comes late but produces adaptive responses specific to the pathogen. Adaptive immune reactions create the classical immune “memory” as the individual is exposed to foreign materials throughout life.

Primary lymphoid organs such as bone marrow and thymus are where the immune cells originate and/ or differentiate. Innate immune cells such as monocytes differentiate into macrophages in the tissue of residence. Adaptive immune cells include T and B cells; innate immune cells are monocytes, macrophages, neutrophils, dendritic cells, mast cells, eosinophils, basophils, natural killer cells, etc. The secondary lymphoid organs are the lymph node, spleen, and payer's patches in the gut. They reserve naive lymphocytes and activate them when necessary.

Different immune cells have distinct functional properties. For example, B cells function to produce antibodies. There are two primary types of T cells. Cytotoxic T cells (CD8+ cells) and T helper cells (CD4+ cells) kill the infected cells and secrete cytokines to coordinate the immune function, respectively. Helper T cells regulate most of the adaptive and innate immune functions. In general, leukocytes communicate and coordinate the immune system through the cytokines such as Interleukins (IL-1, IL-2, IL-3 etc.), Interferons (type I, II and III) and other chemokines such as C-X-C motif chemokine ligand 10 (CXCL10), C-X-C motif chemokine ligand 1(CXCL1), C-X-C motif chemokine ligand 8 (CXCL8) etc.

2.2 Innate immune system and Inflammation

The innate immune system includes physical and chemical barriers and cellular defences. Physical barriers are the skin and mucous membranes in the respiratory and gastrointestinal surfaces. Chemical barriers are antimicrobial peptides and enzymes secreted in the secretions and the acidic pH in the stomach. Cellular defences include phagocytosis, antigen presentation to the adaptive immune cells, producing cytokines and chemokines and killing infected cells.

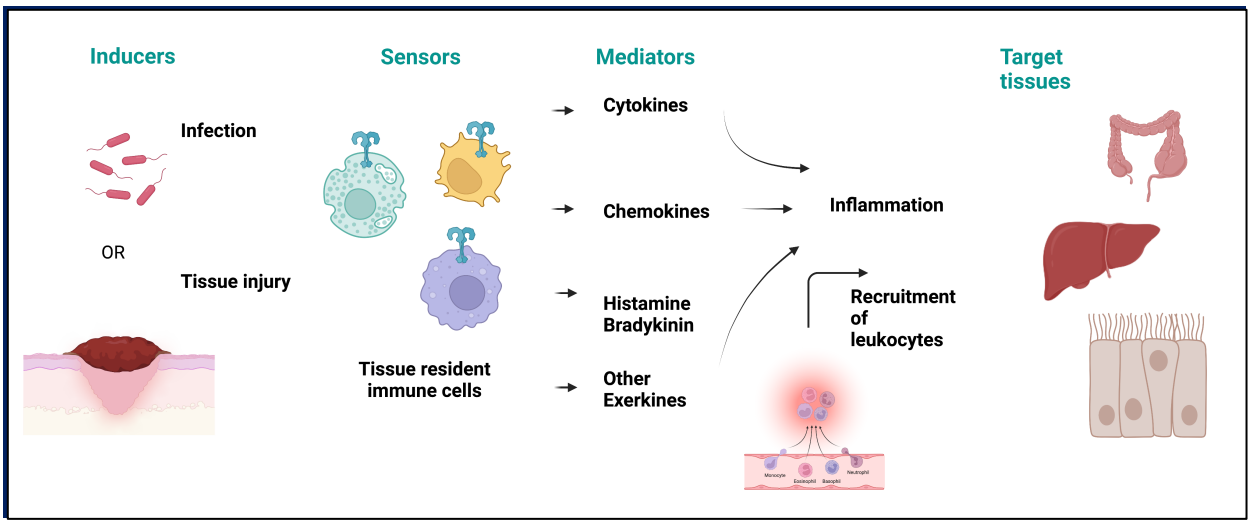


Fig. 2.1: Components of an inflammatory response: An inflammatory response comprises four components: inducers, sensors, inflammatory mediators, and cells that perform the action and the target tissue. Inducers are inflammatory stimuli. Sensors are PRRs present on innate immune cells. These cells release inflammatory mediators such as cytokines, chemokines, and other biomolecules through various intracellular signalling pathways. They regulate inflammation by recruitment of leukocytes to the site and enhancing their function in inflammation at the target tissues. *Created with Biorender.com"*

Inflammation is a tightly regulated innate immune response mounted by the immune system in response to PAMPS or DAMPs.[1–4] Acute inflammation is clinically identified by swelling, redness, pain, and loss of function.[2] An inflammatory response comprises four components: inducers, sensors, inflammatory mediators, and the cells that perform the action and the target tissue. Multiple forms of each component are present, and depending on the pathogen or inflammatory inducer, distinct combinations of these components will be activated to produce the necessary outcome (Fig. 2.1).

Several signalling pathways mediate the cellular responses, beginning with sensing PAMPs or damage associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs), leading to the activation of various transcription factors, including Nuclear factor kapa B (NF-κB), Interferon regulatory factors (IRFs) and Activator Protein-1 (AP-1), which drive the expression of

proinflammatory cytokines and chemokines.[17] Major PRRs involved in the inflammatory response are Toll-like receptors (TLRs), NOD-like receptors (NLRs), retinoic acid-inducible gene (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs) and AIM2 like receptors (ALRs).[18]

TLRs are membrane-bound receptors in the plasma membrane (TLR1, TLR2, TLR4, TLR5, TLR6) or endosomes (TLR3, TLR7, TLR8, TLR9). TLRs in the plasma membrane recognize extracellular pathogens and PAMPs such as protein, flagellin, or lipid components of pathogens and DAMPs. TLR4 is well-characterized among other TLRs and recognizes the lipopolysaccharide (LPS). Endosomal TLRs recognize single-stranded/double-stranded RNA (ssRNA or dsRNA) and CpG unmethylated DNA.[19,20] RLRs such as RIG-I-like receptors or MDA5 receptors recognize dsRNA. [19,20]

These innate immune receptors are present in various cells, including monocytes and macrophages, and respond to most infections.[21] These responses can be divided into phases: homeostasis, proinflammatory response, restoring homeostasis and forming innate immune memory or trained immunity under certain conditions.

Viral infection activates RLRs or a specific subset of TLRs such as TLR 3, 7,9 and activates mainly IRF transcription factors to produce interferon-mediated antiviral responses. Bacterial pathogens bind to TLRs and induce the production of proinflammatory cytokines and chemokine. Cytosolic RNA sensors include RIG-I and melanoma differentiation-associated protein 5 (MDA-5), receptors of viral RNA or synthetic dsRNA. Downstream of RIG-I and MDA-5 signalling, activation of IRFs and NFκB is essential for the induction of gene expression of IFN Is. STING is an ER-associated cytosolic sensor responding to DNA from pathogens, including viruses and bacteria (Fig. 2.2). Functional TLRs form homo or heterodimers upon ligand binding and recruit adapter proteins such as myeloid differentiation primary response protein 88 (MyD88) or TIR

domain- containing adaptor protein inducing IFN-β (TRIF) to recruit and activate downstream signalling proteins (Fig. 2.2). All TLRs except TLR3 recruit MyD88 as an adaptor protein. TLR3 and TLR4 recruit TIR-domain-containing adapter-inducing interferon-β (TRIF). Depending on the adaptor protein recruited, various kinases and ubiquitin ligases are recruited and activated to initiate signalling pathways. The signalling pathways eventually lead to the activation of transcription factors such as NF-**κ**B, Mitogen-activated protein kinases (MAPKs) and IRFs that induce the expression of proinflammatory cytokines, chemokines and IFN I synthesis, etc.[16–18] This study focused mainly on two TLR signalling pathways TLR3 and TLR4. (Fig. 2.2) TRIF signals through Tumor necrosis factor receptor (TNFR) associated factor-3 (TRAF3) or Tumor necrosis factor receptor (TNFR) associated factor -6 TRAF6. MyD88 recruits TRAF6 and leads to the activation of NF-**κ**B and MAPK signalling through receptor-interacting protein kinase (RIP kinase) and Transforming growth factor-β-activated kinase 1 (TAK1) complex. TRAF3 recruits NF-kappa-B essential modulator (NEMO) and IKK-related kinases TBK1 and IKKepsilon (IKKi), resulting in the activation of IRF3 and IFN I production (Fig. 2.2). Parasitic infection leads to the production of histamine, Interleukin-5 (IL-5), nterleukin-4 (IL-4), and nterleukin (IL-13) by eosinophils and mast cells. (Fig. 2) Allergens can also produce the same response as parasitic infections. However, in the case of sterile inflammation, such as tissue injury, inflammation will repair the tissue damage and restore homeostasis. [19,20,22,23]

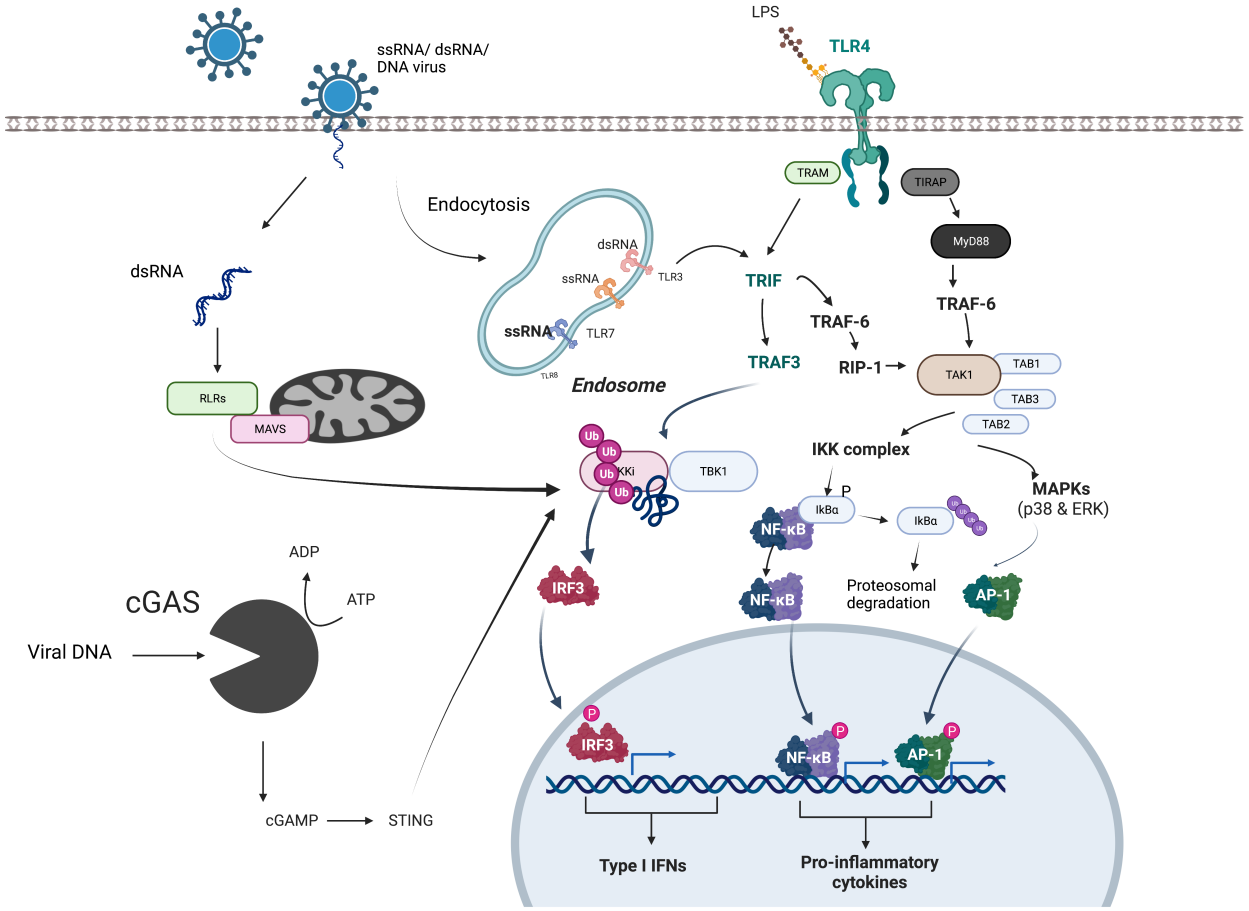


Fig. 2.2: Inflammatory Signalling pathways. TLR4 recruits MyD88 through TIR Domain Containing Adaptor Protein (TIRAP) which recruit and activate TNFR-associated factor 6 (TRAF6). TRAF6 recruits TGF-β activating kinase 1 (TAK1) and its binding proteins such as TAB1, TAB2, and TAB3. TAK1 and IKK complex (IKKα, IKKβ, and NEMO) activation leads to NF-**κ**B and MAPK pathway. TRIF recruits and activates TRAF3 or TRAF6. TRAF6 leads to NF-**κ**B and MAPK Signalling and TRAF3 leads to the phosphorylation and dimerization of IRF3, resulting in the induction of IFN I family cytokine production (i.e. IFN-α and IFN-β). Similarly, RLRs (RIG I-like receptors and MDA5) bind to dsRNAs and activate IRF3 through the IKKi-TBK1 complex, resulting in IFN I responses. STING is a cytosolic DNA sensor that recognizes cyclic-GAMP molecules produced from cytosolic DNA by the cGAS enzyme. This recognition also leads to IFN I response through STING receptor. *"Created with Biorender.com"*

Thus, these inflammatory mediators act on target tissues and cells to produce a coordinated inflammatory response that clears the infection, repairs the damaged tissues, and resolves on its own. Typical local outcomes are vasodilation of blood vessels, recruitment of leukocytes such as neutrophils and monocytes to the site, inducing tissue-resident and recruited immune cells, etc.

When the cytokines such as interleukin-1 (IL-1), interleukin (IL-6) and Tumour necrosis factor (TNF) are secreted in large amounts, they act systemically on tissues such as the liver, bone marrow, fat, muscle and hypothalamus. This results in systemic inflammation and leads to sickness behaviour.[24,25]

2.3 Resolution of inflammation

Once the infection or injury is under control, inflammatory mediators induce suppressive mechanisms to resolve inflammation. This involves a coordinated feedback mechanisms that promote tissue repair and restore homeostasis. This includes down regulating the production of proinflammatory mediators and upregulating the anti-inflammatory mediators such as IL-10, TGF-β, pro-resolving lipid mediators such as lipoxins, resolvins and protectins that promote clearance of immune cells, promote tissue repair.[26–28]

2.4 Acute vs chronic low-grade inflammation

Acute inflammation is clinically identified by swelling, redness, pain, and loss of function.[2] Endothelial cell-derived prostaglandin I2 (PGI2) and leukocyte-derived histamine and bradykinin cause vasodilation and intravascular hydrostatic pressure-induced extravasation. This results in increased blood flow and leakage of body fluid with proteins into the interstitial space of the affected tissues which causes redness and swelling, respectively.[2,4] Furthermore, inflammatory cytokines and chemokines induce the recruitment of leukocytes and sustained swelling. Endothelial cells possess the receptors for these cytokines. For instance, binding of inflammatory

cytokines such as IL-1 and TNF-α to their corresponding receptors (IL1-R1, TNFR1) causes the expression of endothelial cell adhesion molecules such as leukocyte adhesion molecule E-selectin (CD62E), intercellular adhesion molecule (ICAM-1; CD54), vascular cell adhesion molecule (VCAM; CD104) and other monocyte recruitment factors such as chemokine ligand 2 (CCl2) and C-X3-C Motif Chemokine Ligand 1 (CX3CL1). Thereby increasing the significant recruitment of leukocytes to the site, further amplifying and progressing the inflammatory response.[4,29] Moreover, locally secreted cytokines will enter the circulation and act systemically to enhance the inflammation.[2]

However, this increase in inflammatory mediators will subside when the infection or injury is resolved. Chronic low-grade inflammation occurs when these inflammatory mediators are still increased about two to three-fold persistently. The cause of stimulation for this low-level cytokine release is not well defined; however, it is believed that adipose tissues might be the source.[30,31,]

2.5 Dysregulated inflammation

Persistent or dysregulated inflammation is associated with severe diseases, including cancer, cardiovascular disease, type II diabetes, obesity and autoimmune disorders.[25,32–35] Excessive pathological inflammation is also reported in COVID-19 patients, resulting in lung damage and serious outcomes in patients with chronic inflammatory conditions.[34,36–38] Although the triggers are different for sterile and infection-induced inflammation, both responses converge in similar innate immune pathways. A complex of endogenous signals determines when to enhance, dampen, or resolve the inflammation based on the nature of the initial signals and the ongoing cue.[39] Tissue-resident and bone marrow monocyte-derived macrophages play vital roles in inflammation and tissue repair. [40–44]

2.6 Role of macrophages in inflammation

Monocytes and macrophages play pivotal roles in inflammatory responses. They are an essential part of the innate immune system.[45] Whole blood cells are composed of 5-10% bone marrow-derived monocytes with a life span of 1-3 days.[46,47] Macrophages are strategically distributed throughout the body in every tissue. Under a steady state, many tissues constitute embryonically derived self-renewable macrophages seeded from the yolk sac or fetal liver. However, tissues such as heart, skin, and gut macrophages are replaced by bone marrow-derived macrophages after birth.[48,49] Macrophages participate in initiating, maintaining and resolving inflammation, tissue repair and regeneration, restoring homeostasis, regulating adaptive and innate immune responses, etc.[50,51] They are the first line of defence against infection or tissue injury. Additionally, Macrophages play essential roles in tissue development, surveillance and pathologies such as fibrosis, cancer and other inflammatory diseases.[42,52] We can group macrophages' function in immune response into three: immune regulation by producing various inflammatory mediators, antigen presentation to T cells, and clearance of infection or tissue damage through phagocytosis.[53] Monocytes migrate from the blood into tissues and differentiate into macrophages. During a steady state, they differentiate into tissue-resident macrophages, but during inflammation, depending on the stage of inflammatory response at the site of migration, they transform into various phenotypes.[50]

2.7 Immunometabolism of macrophage polarization

Macrophages are well known for their heterogenicity and plasticity. They can attain various phenotypes in tissues depending on the tissue type, location of residence, environmental cues, pathophysiologic conditions, etc. This permits the macrophages to play diverse functions throughout the different stages of inflammation, from initiation to tissue repair.

In broad terms, they were classified into two groups: classically activated macrophages (M1-like), which perform pro-inflammatory roles, and alternatively activated macrophages (M2-like), which are anti-inflammatory. However, it was later established that M1/M2 dichotomy contradicts the macrophage plasticity; a complex spectrum of activated macrophage phenotypes exists (Fig. 2.3).[54,55]

A metabolic switch during macrophage phenotype conversion is well known. M1-like macrophages undergo metabolic reprogramming similar to the Warburg effect in cancer cells. The cell's major energy source becomes glycolysis; oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) are downregulated (Fig. 2.3). FAO is downregulated via downregulating carnitine palmitoyl transferase I (CPT-1), a mitochondrial enzyme that transports fatty acid into mitochondria for FAO. Citrate-derived metabolite malonyl Co-A produced in the cytoplasm at a high rate under inflammatory conditions can also inhibit the function of Carnitine palmitoyl transferase I (CPT-1). Fatty acid synthesis and the pentose phosphate pathway are upregulated, facilitating cell growth and functioning during inflammation.[56–58]

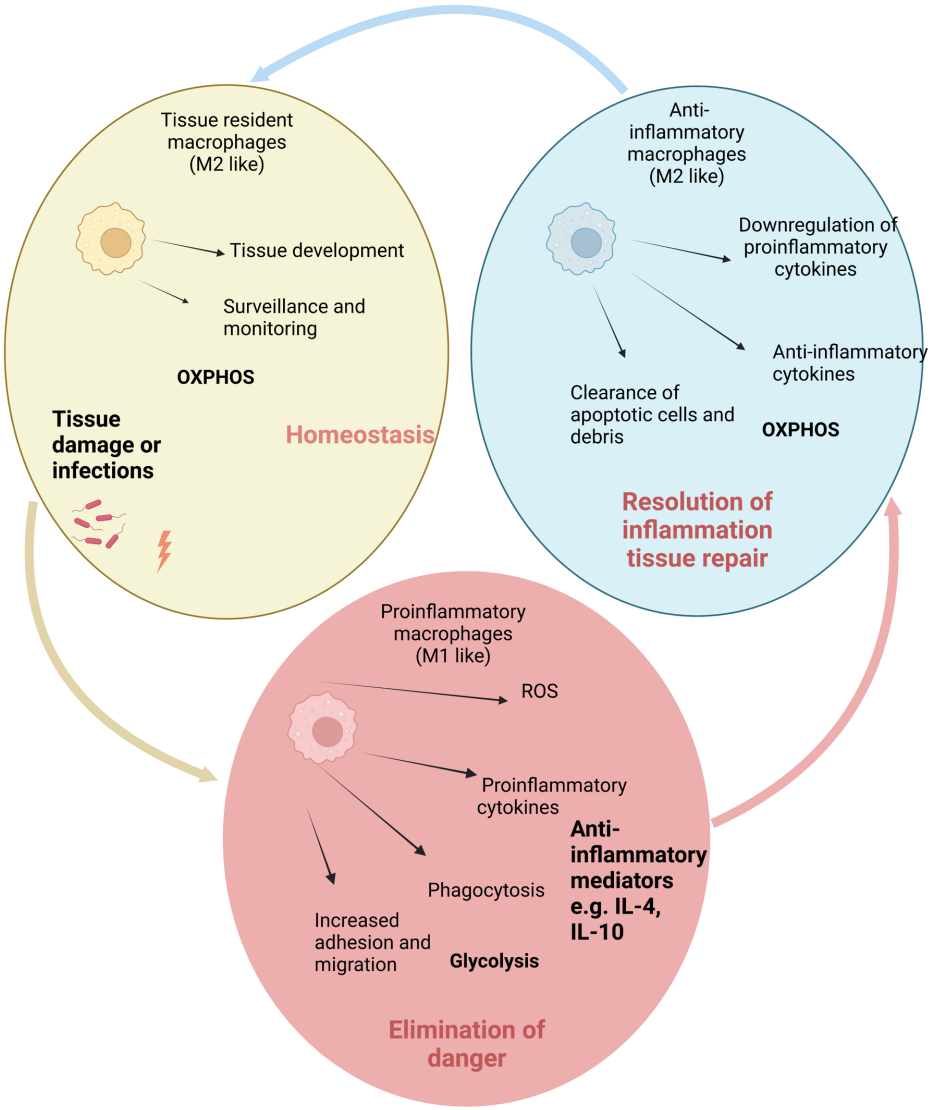


Fig. 2.3: Polarization spectrum of macrophages. During inflammation, macrophages are polarized from the 'M2-like state to the pro-inflammatory M1-like state. This entails a metabolic and transcriptional shift in these macrophages. During homeostasis, macrophages stay in an M2-like state where OXPHOS is the predominant energy pathway. At homeostasis, these macrophages perform functions for tissue development and surveillance (yellow circle). Upon infection or injury, they polarize into an M1-like proinflammatory phenotype. They perform multiple functions, including producing proinflammatory cytokines and ROS and clearing the threat via phagocytosis (Orange circle). As the inflammatory state move towards resolution, anti-inflammatory mediators such as IL-4 and IL-10 polarize them again towards an M2-like phenotype where they are involved in inflammation resolution and tissue repair (Blue circle). Thus, macrophage polarization is a complex spectrum and consists of various phenotypes performing

different functions based on the stage of inflammation they are participating. *"Created with Biorender.com"*

The TCA cycle undergoes two breakpoints in conversion to M1-like macrophages. The first break occurs at isocitrate dehydrogenase (Fig 2.4). This is achieved by downregulating the isocitrate dehydrogenase and upregulating the citrate carrier (CIC; Slc25a1) which results in the accumulation of citrate in the cytosol. Mitochondrial membrane protein CIC is involved in transporting the citrate to the cytoplasm. In the cytoplasm, citrate is converted into acetyl-CoA and oxaloacetate by the ATP citrate lyase (ACLY) enzyme. Acetyl-CoA is used in lipid biosynthesis, which is essential for the cells' growth and functioning under pro-inflammatory conditions. Furthermore, NADPH produced during this conversion of citrate by ACLY enzyme will also be used in nitric oxide synthase (iNOS) catalyzed NO production and NADPH oxidase catalyzed reactive oxygen species (ROS) production in the cytosol. In addition, inflammatory stimuli upregulate the expression of iNOS, which catalyzes the conversion of arginine to citrulline.[59,60] These reactive oxygen and nitrogen species are generally produced as bactericidal components in pro-inflammatory macrophages. However, NO produced in this process disrupts the ETC by nitrosylating iron-sulphur proteins present in electron transport chain (ETC) (e.g., Complex I, cytochrome c oxidase), which will result in mitochondrial ROS (mtROS) generation (Fig. 2.4). [60–62]

The other break occurs at the step of succinate dehydrogenase (SDH), a TCA cycle enzyme and the complex II in the ETC that converts succinate into fumarate (Fig 4). Accumulation of succinate causes hypoxia-inducible factor-1 alpha (Hif1-α) stabilization and activation by inhibiting prolyl hydroxylase (PHD), eventually producing pro-inflammatory cytokines.[57,60,61] Additionally, LPS-mediated activation of macrophages upregulates the Hif1-α expression. This upregulation and activation of Hif1-α involve the mammalian target of rapamycin (MTOR).

MTOR is highly active, helping to meet the high metabolic demand in stimulated macrophages by increasing Hif1- α expression and activation. Increased activity of Hif1- α increases the expression of its target genes, such as Glucose transporter 1 (GLUT1) and glycolytic enzymes. Thus, upregulation and activation of Hif1-α and succinate-mediated stabilization favour the metabolic shift towards glycolysis during inflammation.[60,63,64] Thus, mitochondria and metabolic alterations are vital to macrophage polarization (Fig 2.4).

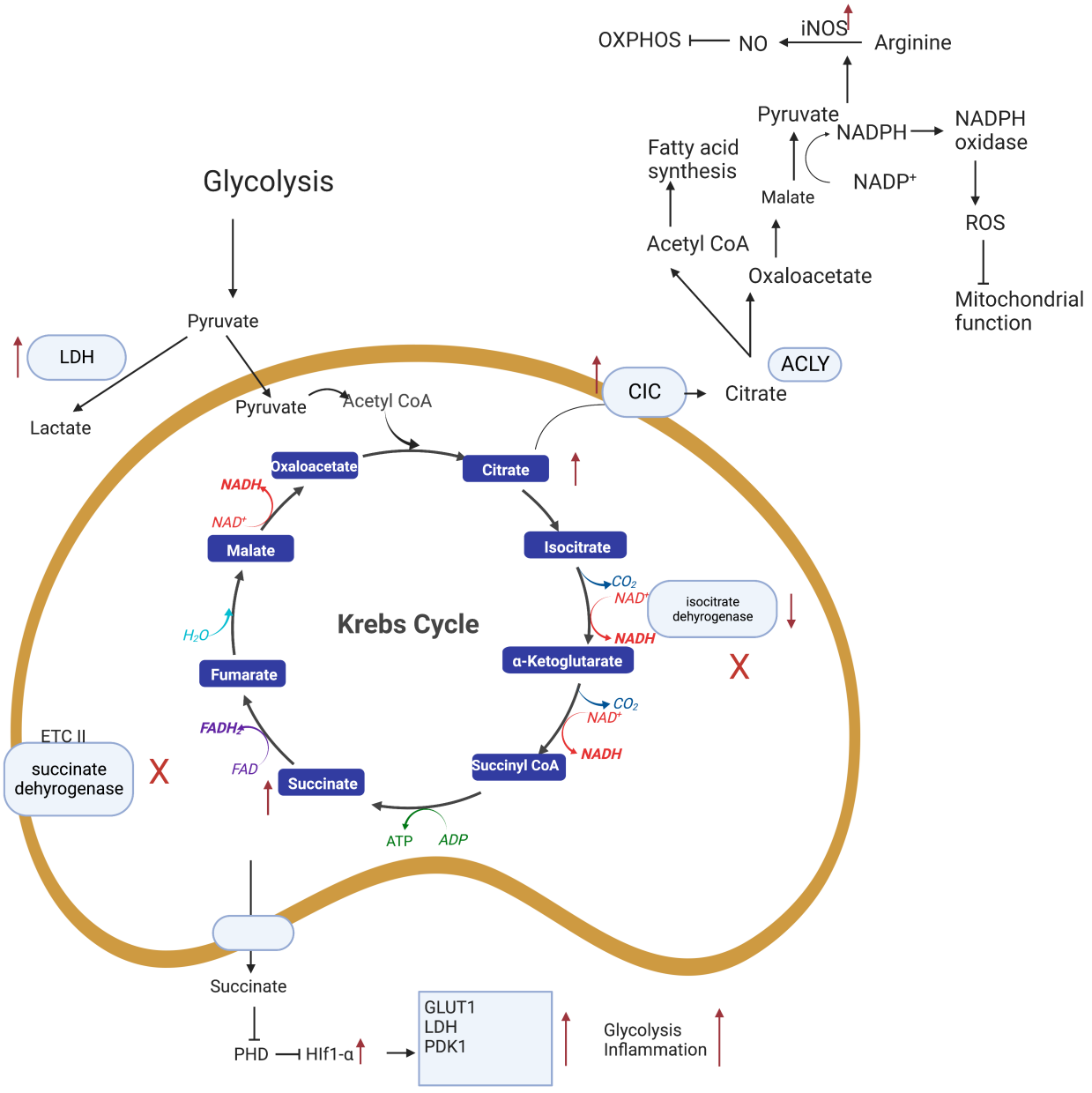


Fig. 2.4: Role of the metabolic switch in ETC damage, mtROS generation, and inflammation. Inflammatory stimuli cause a metabolic switch in immune cells. The TCA cycle undergoes two breaks under inflammatory conditions. The first break occurs at isocitrate dehydrogenase, and the second occurs at succinate dehydrogenase. Accumulated citrate will be transported to the cytosol via citrate carrier CIC and metabolized to acetyl-CoA and oxaloacetate. Further processing of these metabolites and increased expression of iNOS will lead to reactive oxygen and nitrogen species, which will cause damage to mitochondria and the electron transport chain (ETC). Accumulation of succinate will increase Hif1-α activation, leading to increased glycolysis. Under inflammatory conditions, Lactate dehydrogenase (LDH) is upregulated, reducing pyruvate utilization in the TCA cycle. *"Created with Biorender.com"*

Four important immunometabolism regulators in immune cells are 5' AMP-activated protein kinase (AMPK), mechanistic target of rapamycin (mTOR), Peroxisome proliferator-activated receptors-γ (PPARγ) and HIF-1α. AMPK is an energy sensor activated at low Adenosine triphosphate (ATP)/ Adenosine monophosphate (AMP) levels. On the other hand, AMPK negatively regulates the PI3K-Akt-mTOR pathway. Several studies have shown that AMPK signalling is activated following exercise in muscle[65], the data is limited on immune cells.[66]

mTOR is the master regulator of metabolism, comprising two sub-complexes, mTORC-1 and mTORC-2. mTORC-1 is another energy sensor, induced by amino acids or growth factors via PI3K/AKT pathway, and functions in opposition to AMPK.[65,67]

Furthermore, the mTOR-HIF-1α signalling axis is vital in the metabolic switch to glycolysis during immune cells' phenotype conversion, such as macrophages and T cells. In contrast, mTORC-1 PPAR-γ/PGC-1α axis activation is essential for mitochondrial adaptations related anti-inflammatory responses.[68] Thus, the delicate balance in these pathways is essential to establish balanced polarized states of these immune cells, thereby, the balanced inflammatory cells.

However, the role of mTORC-1 on macrophage phenotype conversation to pro or anti-inflammatory is still unclear.[69,70]

2.8 Exercise in Health and disease

Physical activity is known as physical exercise when it has an objective and becomes planned, repetitive, and structured. These include household work, walking, cycling, or other leisure activities such as swimming, dancing, or playing. Exercise perturbates homeostasis in various ways [71], bringing adaptations to many tissues.

Physical exercise can be either acute or chronic. When an acute bout of physical activity is repetitive, it is known as chronic exercise or exercise training. Overall, the effect of exercise depends on the exercise modality and intensity, usually expressed as the percentage of maximal oxygen uptake by an individual ($VO2_{max)}$.[72–74]

Several works of literature have shown that exercise has numerous health benefits in preventing or treating at least 35 chronic diseases, including type 2 diabetes, cardiovascular disease, non-alcoholic fatty liver disease, auto immune diseases, and certain cancers. Exercise has systemic effects that result in various adaptive responses contributing to health benefits. Although most of the literature examined skeletal muscles, exercise also mediates adaptations in other tissues.[75,76] Perhaps, crosstalk between these tissues upon exercise-mediated perturbations might occur.[75,76]

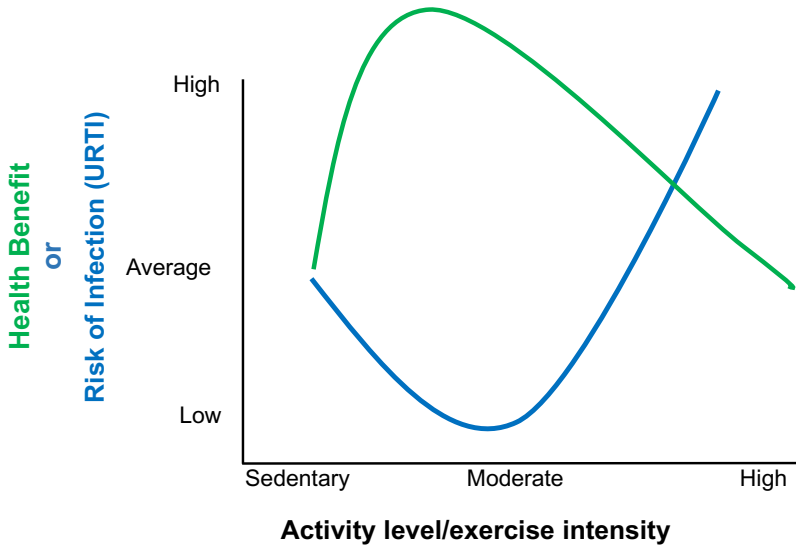
A significant body of literature reported that moderate physical exercise could treat or prevent many health conditions, such as cardiovascular diseases, insulin resistance, other metabolic diseases, immune-related diseases, etc.[77–80,81,82] However, high-intensity exercise is shown to course adverse effects.[83–85] Studies showing the effect of exercise intensity and duration on health outcomes are extensively reviewed by Nieman et al. in multiple reviews.[86–90]. In 1994 Neiman et al. proposed a J-shaped model for opportunistic infection risk vs. exercise intensity. He proposed this model by summarizing studies on Upper Respiratory Infection Risk (UTRI) in athletes involved in heavy training, ultramarathon events and moderate exercise training.[86,89] This

model was challenged later by many studies who claimed that these studies relied on self-reported URTI symptoms that were clinically not confirmed.[91] Indeed, another study later confirmed that only 30% of the self-reported infections following heavy training or mass participation events such as ultra-marathon had a positive laboratory diagnosis (Fig 2.5).[91,92]

Furthermore, studies have shown that very elite intense trainers are less prone to illness, suggesting that they adapt well to the training demands. This leads to an S-shaped model for infection risk vs. exercise intensity.[93] Thus, a clear relationship between exercise intensity and duration vs. immune system functionality is unexplored.[94] A clear distinction between moderate and high-intensity exercise regimens must be defined to prescribe exercise therapies clinically.

There is a belief that high-intensity exercise impairs the immune response, leading to an "open window" for the risk of infection. This may occur through acute functional impairment of various parts of the immune system. However, there is no reliable evidence for this dogma.[69–71] Considering high-intensity exercise, few studies investigated the effects on immune cells such as macrophages and T cells. They looked into peritoneal macrophages and T cells from peripheral lymphoid organs such as lymph nodes and reported metabolic and functional alterations in these cell types.[98,99] Another study on peripheral leukocytes showed impaired mitochondrial function and inflammation upon acute high-intensity exercise.[100] Furthermore, systemic reviews by Massett et al. 2021 and Cerqueira et al. 2020 nicely reviewed the papers that examined exercise immunology of different exercise models with various intensities and duration.[101–103]

The increased inflammatory response and impaired immune function resulting from high-intensity exercise could be associated with the body's adaptation to heavy exertion. If the body has not adapted to the exercise-associated trauma with time, it will still be vulnerable to immune responses following high-intensity training.



Modified from Neiman, Med Sci Sports Exerc 1994; and Schefferand Latini 2020

Fig. 2.5: Physical exercise intensity and health benefit or risk of infection (UTRI)

2.8.1 Exercise, infection risk and immune function

The positive correlation between repeated moderate-intensity exercise and the immune system's function against infection is well appreciated. Repeated moderate exercise-induced adaptations include reducing oxidative stress, increasing anti-oxidative capacity and improving the metabolic capacity of the cells, thereby improving the cell's immune function and inflammatory state.[38,104–108] Conversely, high-intensity exercise is reported to increase inflammatory response and impair immune functions against infections.[86–90]. However, most of these studies only examined the immediate effects on immunity, while the prolonged effects of exercise on inflammation are still unexplored.[107]

Moreover, an immune response to an infection or injury is a complex array of interconnected events. This involves multiple coordinated adaptive and innate immune system responses. Therefore, overall immune competency may not directly reflect the level of inflammation or other natural immune mechanisms.[109,110]

Exercise-mediated health benefits and an individual's responses to exercise may vary if they have pre-existing chronic inflammatory conditions. Thus, generalized exercise therapies or the patients meeting the exercise requirements are not always possible for various reasons. Designing precise exercise therapies or designing exercises mimicking pharmaceuticals to treat or prevent various chronic diseases requires an extensive understanding of exercise biology.

2.8.2 Anti-inflammatory effects of exercise

Exercise has been implicated in altered cytokine profiles and leukocyte mobilization, two essential factors in inflammation.[111–115] Exercise alters body homeostasis by changing energy demand, O_2 availability, tissue stress or damage etc. These changes result in the release of various molecules into the circulation, including myokines, metabolites, hormones, cytokines, chemokines, specific peptides, nucleotides, etc. Collectively they are named "Exerkines", any biomolecules secreted into the circulation upon exercise.[9]

A bout of exercise induces inflammatory responses in muscle, but the ultimate effects are anti-inflammatory. For example, exercise-induced IL-6 release in muscle is well known. The effects of IL-6 in muscle upon exercise depend on the level of exertion; it can be inflammatory or anti-inflammatory.[116] IL-6 increases the release of anti-inflammatory mediators such as IL-10 and IL-1RA.[117] IL-10 inhibits pro-inflammatory cytokine productions, and interleukin receptor agonist (IL-1RA) inhibits IL-1β signaling.[118,119] Thus, acute bouts of exercise-induced anti-inflammatory effects can be mediated in part by IL-6 release along with other mechanisms. Future studies investigating cell type-specific mechanisms, especially in immune cells in combination with different exercises, are needed.

Several studies examined the effects of exercise on various tissues other than muscle. For example, a study on rats recently reported that chronic exercise enhances the immune system. They showed that the overall proliferative capacity and the splenocytes architecture are altered after the exercise.[120] Another study recently reported that endurance exercise training in mice activates IL-13 signalling in muscle; IL-13 in mice increases the gene expression that favours fatty acid oxidation and the expression of electron transport chain complexes via and Stat-3.[121] Another study on rats recently reported that an intense exercise training altered the inflammation-associated biomarkers such as phagocytic activity, ROS production and cytotoxic activity of the NK cells.[122]

Furthermore, several studies have shown exercise-mediated modulation of monocyte infiltration or macrophage function in adipose tissue of obese patients. For example, a study showed physical fitness reduced proinflammatory monocyte infiltration in obese patients.[123] Another study in mice showed chronic exercise is associated with macrophage anti-inflammatory phenotype in adipose tissue.[124] In addition, a study in the rat reported both interval and continuous aerobic training combined with a high-fat diet increased the anti-inflammatory macrophages in adipose tissue.[125]

Studies have also reported the anticancer effects of exercise. For example, a meta-analysis by Moore et al. 2016 showed that increased leisure-time physical activity (self-reported) is associated with decreased rick across 26 types of cancer even after normalizing to body mass index (BMI).[126] Furthermore, tumour incidence and growth have been reduced 60% in voluntary wheel-running mice than in sedentary mice across five tumour models. The study showed that IL-6 and adrenaline-induced natural killer cells (NK cells) mobilization and infiltration are associated with this observation.[113] Another study reported metabolites released during exercise altered the T cell profile, which improved their anticancer properties in mice.[127,128] Catecholamine release is shown

to cause adaptations in peritoneal macrophages in mice upon exercise.[129–131] However, the mechanisms behind these beneficial anti-inflammatory or anti-cancer effects are poorly defined.

2.8.3 Exercise mediated adaptations

2.8.3.1 Mitochondrial adaptations upon exercise

Macrophages, during homeostasis, rely on oxidative phosphorylation (OXPHOS) for energy as they don't have high energy demand. Inflammatory response by macrophages involves a metabolic switch, proinflammatory signalling and secretion of essential cytokines. Some of which require the NLR family pyrin domain containing 3 (NLRP3) inflammasome activation for their secretion.[88] Mitochondrial damage occurs during the metabolic shift and plays a significant role in inflammasome activation and macrophage phenotype conversion. The proinflammatory phenotype of the macrophage is called M1-like; the phenotype that exists either in homeostasis or performs inflammation resolution and repair is known as M2-like macrophages (Fig. 2.3).[57,60,61,132]

However, excessive mitochondrial damage may result in uncontrolled inflammation and cell death. Cells need mechanisms to maintain the mitochondrial network. The mechanisms involved in this maintenance are mitochondrial biogenesis mediated through PGC1-α signalling and mitophagy.[133–135] One well-known lifestyle factor impacting this process is exercise.
Following the seminal work that discovered the increased mitochondrial proteins in the muscles of treadmill trained rats[136], several studies have shown mitochondrial adaptation related to exercise in skeletal muscle. [137–139]

A couple of recent studies have also shown that exercise promotes muscle mitophagy. [140–144] Another recent elegant study on mice relating to Parkinson's disease has shown a strong inflammatory phenotype in Parkin$^{-/-}$ and Pink1$^{-/-}$ mouse models following exhaustive exercise. They further showed that the absence of STING completely rescues the observed inflammatory

phenotype, a cytosolic DNA receptor that regulates IFN I response.[145] However, evidence for mitochondrial adaptation in immune cells upon exercise is not prevalent. Taken together, looking at mitochondrial turnover, oxidative stress and other mitochondrial dysfunction-associated parameters to study the effect of exercise on inflammatory cells such as macrophages will be useful.

2.8.3.2 Trained immunity and epigenetic modifications

Immune memory has now been linked to both adaptive and innate immune systems. The mammalian immune system has two different arms, adaptive and innate immune systems. The innate immune response is faster, but broadly specific and keeps the infection under control and coordinates the adaptive immune system, which mounts a highly specific response to the particular infection.[146] Though the mechanisms by which they produce and maintain the memory are different, they result in beneficial effects for the host. Trained immunity, a concept adopted recently, occurs when long-lasting functional reprogramming of immune cells, including macrophages, happens due to epigenetic and metabolic rewiring following infection or injury. This can result in either an enhanced secondary response or a tolerogenic response.[147–149] This may depend on the nature of the primary stimulus, such as infection, tissue injury, and individual's ability to respond to the secondary infection. Tolerance is a process that dampens the immune system's response to protect organs from failure and maintain tissue homeostasis.[146–148]

Eukaryotic DNA is packed in chromosomes, enabling the cells to contain vast genetic content. Chromosomes are compact chromatin structures that consist of repeating nucleosome units. Nucleosomes are DNA of 147 bps wrapped around histone octamers. Chromatin structure plays essential roles in transcriptional regulation and gene silencing. Open chromatin regions facilitate transcription factor accessibility, while the highly compact structure involves silencing.[150–152]

Epigenetic modifications influence the chromatin structure and involve various epigenetic enzymes, their activators, and upstream signalling pathways that provide tight control over these processes. DNA methylation at cytosine residues generally represses the transcription via either modifying chromatin accessibility or by impairing the transcription factor binding to its response elements.[153] Histone posttranslational modifications substantially impact chromatin structure and transcriptional regulation.[154–156]

Epigenetic modifications are heritable through cell division. These modifications include DNA methylation, histone posttranslational modifications such as acetylation, methylation, phosphorylation, ubiquitination, and adenosine di phosphate (ADP) ribosylation and have been reviewed by many literatures.[153,157,158]

DNA methylation acts as an on-off switch for gene expression, and histone modifications such as histone acetylation increase the exposure of promoter regions of coding regions for transcription.[158–162] Chromosome accessibility is essential for transcriptional regulation. Biological factors and lifestyle factors affect the epigenome. Lifestyle factors include diet, exercise, stress, etc.

2.8.3.3 Epigenetic modification via exercise

There is much literature available on exercise-induced epigenetic modifications.[158–166] Notably, a review published in Nature summarized the evidence regarding epigenetic modifications occurring via lifestyle factors that affect metabolic health.[162] Furthermore, exercise-mediated DNA methylation associated with human diseases is well-reviewed in Grazioli et al., 2017. Exercise-mediated histone modifications in the muscle are reviewed by McGee et al., 2009 and McGee and Hargreaves, 2011. However, whether exercise can cause persistent epigenetic modifications in immune cells such as macrophages still need to be studied (Fig 2.6). [159,161,164]

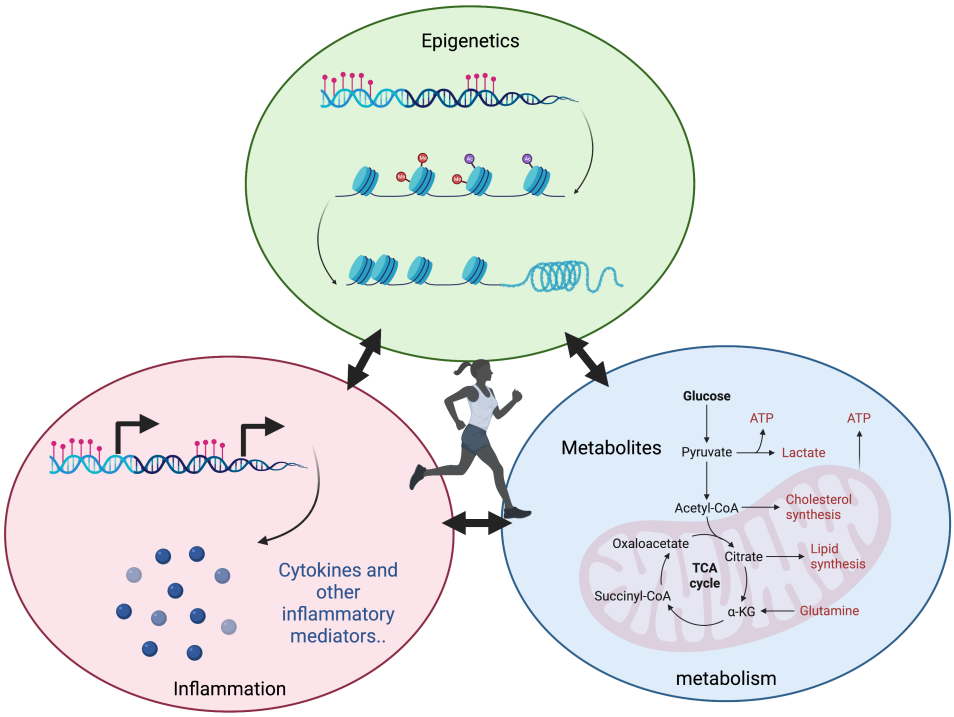


Fig. 2.6: Exercise mediated trained immunity. Exercise induces inflammation and metabolic changes in the immune cells including macrophages. Inflammatory mediators and metabolites secreted upon the long-term exercise training can induce epigenetic modifications by activating epigenetic enzymes. As shown by the arrows, all three alterations can influence each other leads to persistent exercise mediated adaptations. *"Created with Biorender.com"*

2.8.4 What did multi-omics approaches reveal?

Several metabolomic studies have been published on exercise-induced perturbations. Most of the studies used Liquid chromatography–mass spectrometry (LCMS)/MS to identify the altered metabolomes. Gas chromatography–mass spectrometry (GCMS), Ultrahigh-pressure (or performance) liquid chromatography- MS (UHPLCMS), and ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-qTOF-MS) have also been employed in this field. The type of sports population studied were cyclists and runners (both sprinters and marathon runners). Overall, these studies identified numerous metabolites that are different upon exercise. This is reviewed nicely in the paper by Camila A and Sakaguchi et al. 2019.

A longitudinal multi-omics study in 2020 revealed various metabolites and biological processes are altered with acute exercise. The altered mechanisms include inflammation, tissue repair, energy metabolism, growth factor responses and many other regulatory pathways.[167] In addition, the MoTrPAC study group recently identified 35439 analytes modulated upon endurance training in rats. This study used a multi-omics approach, covered 18 tissues and examined multiple exercise durations such as 1, 2, 4 and 8 weeks and the tissues were collected 48 hrs after exercise cessation. However, this study did not provide insights on single-cell platforms, which might offer more mechanistic insights.

Overall, studies on exercise mediated persistent adaptations on immune cells are still lacking. Studies investigating how various exercise intensities affect different immune cells will improve our understanding on how exercise is affecting the immune system directly.

2.9 Interferons (IFNs)

Interferons are classified into three groups: type I, Type II and III interferons (Fig. 7). Type I IFN (IFN Is) are monomeric cytokines consisting of single gene products of IFN-β, IFN-ε, IFN-τ, IFN-κ, IFN-ω, IFN-δ, IFN-ζ and multiple partially homologous IFNαs. To date, we know that there are 17 type-1 interferons in humans (IFN-B, 13 αs, ε, κ, ω τ, δ and ζ) and 18 in mice (IFN-B, 13 αs ε, κ, ω, τ, δ and ζ) and are clustered in the same chromosome, chromosome 9 in human and 4 in mice (Fig. 2.7 & 2.8).[15] All infected cells can produce type I interferons, but plasmacytoid-dendritic cells (pDCs) are specialized producers of type I interferons. In addition to their well-known antiviral and other antimicrobial functions against fungal and parasitic infections, they have been shown to suppress cancer and regulate innate and adaptive immune responses.[168–171] Furthermore, IFN-β and IFNαs are among the first approved protein drugs used against various diseases such as Multiple sclerosis (MS), hepatitis B & C and some cancers.[14,172] All IFN Is bind to the same

receptor complex, which consists of two transmembrane subunits, interferon-α/β receptor (IFNAR1 & 2). Each transmembrane units constitute an extracellular IFN binding domain, a transmembrane region, and an intracellular domain. Type II interferon contains only one member, IFN-γ, predominantly produced by NK-cells and T cells, independent of type I interferons in terms of their functionality and signal transduction (Fig. 7).[173–175] However, IFN-γ involves supporting IFN I functions and mediates cellular immunity by activating macrophages.[176] The recently identified type III interferons IFN-λs (λ1, 2, 3 and 4) are shown to be produced by virally infected cells but limited to the specific cell type based on their expression and receptor availability (Fig. 7).[177–179] Despite their different receptor complexes, the signalling pathways of all three IFNs overlap and act synergistically to coordinate antiviral functions effectively.

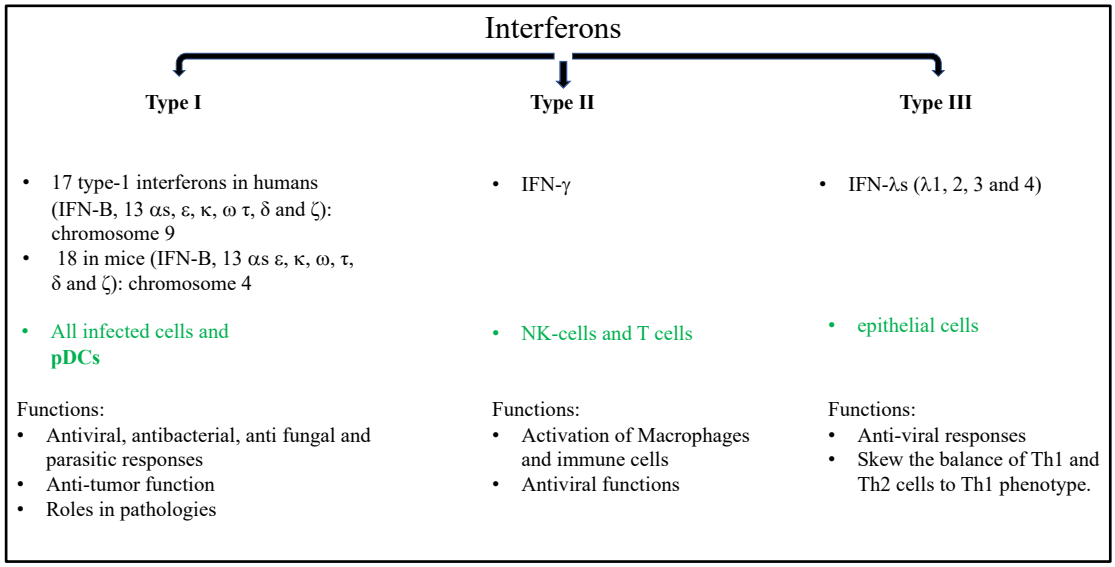


Fig. 2.7: IFN I subtypes, their cellular source and principal functions.

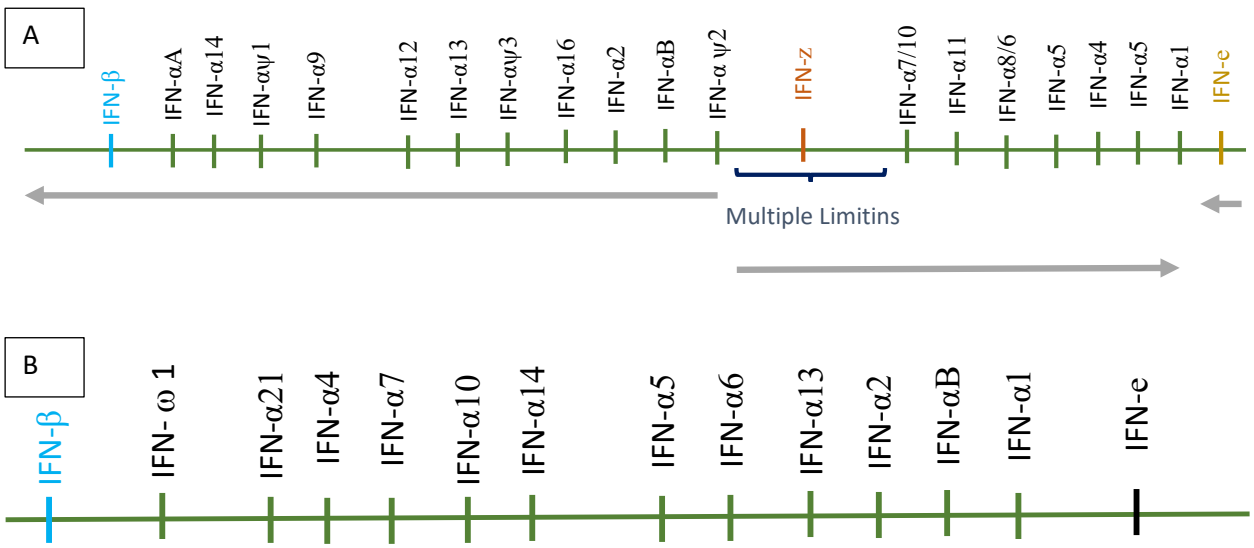


Fig. 2.8 Map of IFN I locus. Grey arrows indicate the transcription direction. Multiple limitin genes (interferon-like cytokine) are in the middle of this gene cluster. A) Mouse, B) Human

2.9.1 The initial wave of Type 1IFN production via PRR signalling

Any infected cells can produce Type 1 IFNs upon microbial product-mediated stimulation of PRRs, including certain TLRs and cytosolic RIG-1, MDA5 STING.[180–182] Subsequent signalling pathways lead to the secretion of IFN Is, and interferon-stimulated genes (ISGs) through the activation of transcription factors IRFs and NFκB. Studies suggest that in most cell types, initial wave induces IFN-β and IFN-α4 in mice (IFN-α1 in humans) (Fig. 2.2).[173,176,183–193,193]

2.9.1.1 IFN I signalling via PRRs: TLRs, RIG-I, MDA5 and STING

As described in section **3.2**, RIG-I and MDA-5 are cytosolic receptors of viral dsRNA or synthetic dsRNA. IRFs and NF-κB activation downstream of these receptors leads to the expression of IFN Is. STING is an endoplasmic reticulum (ER) associated cytosolic DNA sensor. (Fig. 2.2) [18]

TLRs in the plasma membrane recognize extracellular pathogens and PAMPs such as protein, flagellin, or lipid components of pathogens and DAMPs. TLR4 sense the lipopolysaccharide (LPS). Endosomal TLRs (TLR3, TLR7, TLR8, TLR9) recognize single-stranded or double-stranded RNA (ssRNA or dsRNA) and CpG unmethylated DNA.[19,20] RLRs such as RIG-I-like receptors or MDA5 receptors recognize dsRNA. [19,20]

TLR3 and TLR4 recruit TRIF. The signalling pathways eventually lead to the activation of transcription factors such as NF-**κ**B and IRFs that induce the expression of proinflammatory cytokines, chemokines and IFN Is in the early phase. [19,20,22,23] This study focused mainly on two Signalling pathways TLR3 and TLR4. (Fig. 2) TRIF recruits and activates TRAF3 or TRAF6. TRAF3 recruits NEMO and IKK-related kinases TBK1 and IKKi, which leads to the phosphorylation and dimerization of IRF3, results in the induction of IFN I production (Fig. 2.3) [19]

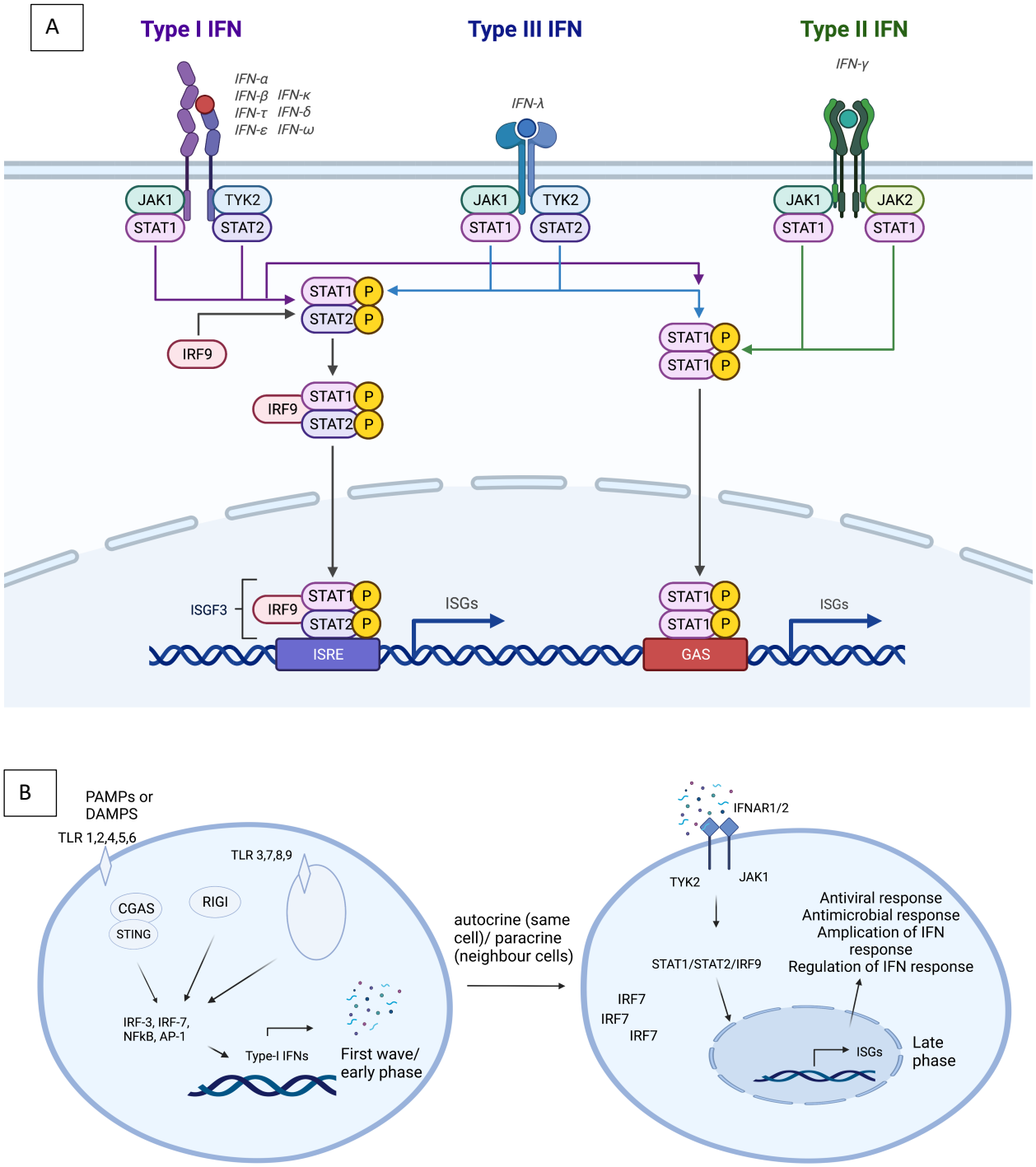


Fig. 2.9: IFN signalling. A) Type I and III IFNs binding to IFNAR or IFNLR, respectively, recruits JAK1 and TYK2, which results in the phosphorylation of the receptors, leading to the phosphorylation and dimerization of STAT1 and STAT2. STAT1/STAT2 dimers then interact with IRF9 to form the ISGF3 complex, which then translocate into the nucleus and binds to ISRE promoter elements to express ISGs. Type III IFNs also follow the same signalling pathway as Type

'sI and induce the expression of ISGs. On the other hand, type II IFN entails homodimerization of STAT1 upon binding to its receptors IFNGR1 and 2. STAT1 homodimers translocate into the nucleus, bind to the GAS promoter elements, and induce ISG expression. B) First and second wave of IFN I signalling. *"Created with Biorender.com"*

2.9.2 The second wave of IFN I production via IFN signalling

IFN Is from the initial wave binds to IFNAR receptors and induce the subsequent wave (Fig. 2.9). This second wave increases IRF7 phosphorylation, producing a positive IFN I production feedback loop (Fig. 9).[194–196] However, IRF7 is constitutively expressed in plasmacytoid dendritic cells (pDCs), specialized in IFN I production.[197] These 1st and 2nd waves of IFN I production occur at 12h and 48h post-infection.[198,199] First wave is crucial for MCP-1-induced monocyte recruitment to the site of infection.[200] IL-18 released by monocytes recruits NK cells, crucial members of antiviral response. NK cells produced IFN-γ (Type II IFN) induces macrophage activation and other antigen-presenting cells (APCs). [176] Other IFN Is, such as IFN-κ and IFN-ε are shown to be restricted to specific cell types such as keratinocytes and epithelial cells. However, immune cells such as macrophages can still upregulate them. IFNε is also shown to be regulated independently of PRRs and IRF signalling; instead, it is shown to be regulated by hormones.[201–206]

IFRAR1 and 2 form a ternary complex upon IFN I binding, which is endocytosed and signalled through their downstream signalling. [207–211] As mentioned previously, IRF3 and IRF7 are strongly upregulated by the first wave of IFN-1s signalling, resulting in cross-phosphorylation and activation of associated tyrosine kinases such as Janus kinases (JAKs) and signal transducer and activator of transcription (STATs) (Fig. 9).

Three of the known four JAK kinases (JAK1, JAK2 and TYK2) are involved in IFN signalling.[212] Type I and III IFNs bind to IFNAR and IFNLR respectively and recruit JAK1 and TYK2, which results in phosphorylation of the receptors, leading to the phosphorylation and STAT1 and STAT2. Interaction of STAT1/STAT2 dimers with IRF9 forms ISGF3 complex, which then translocate

into the nucleus and binds to ISRE promoter elements to express ISGs. (Fig. 3) Type III IFNs also follow the same signalling pathway as Type I and induce the expression of ISGs. On the other hand, type II IFN entails homodimerization of STAT1 upon binding to its receptors IFNGR1 and 2. STAT1 homodimers translocate into the nucleus, and bind to the GAS (Gamma-activated genes) promoter elements which regulate the IFN-II induced gene expression (Fig. 9).[171,173,213–215] JAK1, STAT1 and STAT2 are expressed in all cells, but other STATs are cell-specific.[172]

In addition to STAT dependant pathways, IFN Is can also induce various other signalling pathways and produce diverse outcomes. These pathways include the MTOR pathway, PI3K pathway, P38 (MAPK) and ERK 1/2 pathway.[172,182] They influence IFN I-dependent genes at transcriptional and translational levels. [182,207,216]

As mentioned, the signalling pathways of all three IFNs overlap and act synergistically, adding more complexity to the signalling pathways.

2.9.3 Interferon Stimulated genes (ISGs)

Interferon stimulated/regulated genes (ISGs) are a group of genes known to be regulated during interferon response. A group of signaling proteins that are significantly up or downregulated during IFN responses are defined as ISGs. IFN Is mediate various biological responses through the induction of approximately 2000 IFN-stimulated genes (ISGs). Advances in microarray techniques and high throughput sequencing techniques established the INTERFEROME database, which is an excellent resource for inquiring about IFN responses.[217] Currently, it is known that IFN signalling can regulate about 10% of the human genome. However, it is reported that only 62 genes out of IFN inducible genes are conserved among various animals, suggesting evolutionary importance for these genes. [217,218]

ISGs can be proteins, long-coding RNA and microRNAs.[219,220] This includes all ISGs that interfere directly with the pathogen life cycle or indirectly in the signalling pathways. For example, All IFN I subtypes, PRRs, and many signalling proteins mentioned above, such as JAK2, STAT1 and 2, belong to ISG family [221–226] and are present at baseline.[227] Some others are only expressed during IFN responses. To add more complexity, some transcription factors such as IFRs (1, 3 and 7) and NFκB are also IFN inducible.[228–230] Interestingly, several ISGs are also shown to be repressed during interferon response. This subset of genes is named (interferon regulated genes) IRGs or interferon repressed genes (IRepGs).

2.9.4 What do ISGs do?

Characterizing the mechanisms of identified ISGs is one of the main goals in this field. ISGs perform various functions, which include antiviral, anti-bacterial and anti-parasitic infections and anti-tumour immune responses. Many ISGs are also shown to regulate the immune system by inducing chemokine and cytokine production and enabling cell-to-cell communication. The repressive chemokines involve resolving the IFN responses and restoring homeostasis. They achieve this through their diverse functional properties that involves antigen processing and presentation, apoptosis, cellular differentiation, activation etc.[182]

A successful viral replication cycle includes three main stages. They are 1) attachment, entry, and trafficking 2) Viral protein production and genome amplification 3) viral particle assembly and aggress. In the antiviral responses of ISGs, the mechanism of action or mode of inhibition of viral replication has been defined for many ISGs. For example, Interferon-induced transmembrane protein 1, 2 & 3 (IFITM1,2 & 3) are shown to inhibit the viral entry at various stages of viral replication.[217,231,232]

2.9.5 Are there qualitative differences in IFN I-mediated ISG regulation?

Interestingly, a study in 2020 examined the interferomes for a subset of IFN Is (α1, 2, 5, 8, 14 and β) in pDCs showed a set of 246 ISGs as core ISGs induced by all tested IFN Is. This study also showed that IFN-β induced a broad spectrum of interferons compared to other subtypes. Notably, of 1968 ISGs, many of them were not shared between the tested subtypes and 112 of them were IFN-β specific ISGs. Although this study didn't examine all subtypes, this observation suggests qualitative differences between IFN I subtypes in their responses.[175]

2.9.6 Antiviral responses by IFN Is

The antiviral roles of IFN I are well established, but many unanswered questions remain. Notably, the mechanisms behind differential responses of IFN I subtypes need to be addressed.

Several studies have investigated various viruses on both animal and human models, to uncover the mechanisms behind the differential roles of IFN-β and IFN-α subtypes. This includes Lymphocytic Choriomeningitis Virus (LCMV), human immunodeficiency viruses (HIV), hepatitis C virus (HCV), hepatitis B virus HBV, Chikungunya virus (CHIKV), West Nile virus, Vaccinia virus, Influenza virus, etc.[211]

Following the short-lived initial responses, IFN I level go down to the basal levels despite the elevated viral load. However, even at the undetectable levels of IFN Is, continuous expression of ISGs was evidenced, which was ablated when the IFNAR was blocked. [233–235] The mechanism behind these continuous IFN I responsiveness or ISG production is yet to be studied. However, this could be due to cellular adaptations such as epigenetic changes.

Interestingly, IFNAR blockade in the early stages of infection resulted in immune exhaustion, but blocking in the middle of infection restored the T cells' antiviral properties.[234,236] This suggests

that understanding the kinetics and functional properties of individual Type 1 IFNs subtypes is crucial to understand their complex responses.

This literature review will focus on reviewing the knowledge on the roles of **IFN-β vs. IFN-αs.**

2.9.7 Role of IFN-β vs. IFN-α in viral infections.

Lymphocytic choriomeningitis virus (LCMV)

Lymphocytic choriomeningitis virus (LCMV) is an *Arenaviridae* family negative strand non-lytic virus that causes hemorrhagic fevers.[237] There are two different clones of LCMV; one is LCMV clone 13 (LCMV Cl-13), which is just three amino acids different from the parent strain LCMV Armstrong (LCMV-Arm). The former causes chronic infection, and the latter causes acute infection.[234,238–241]

The role of IFN Is in LCMV infection is also shown by deleting the IFNAR receptor subunit IFNAR1. Loss of IFNAR1 led to increased viral load in the early stage of disease; however, it ultimately showed positive outcomes, including increased clearance of chronic viral infection, decreased levels of negative immune regulators such as IL-10 & Programmed death-ligand 1(PDL-1), improved protective acquired immune responses, improved splenic architecture, etc.[240,240,242]

Interestingly, considering the studies examined the differential effect of **IFN-β vs. IFN-αs**, the IFNAR knockout mouse strain of NZB showed protection against LCMC Cl-13 induced severe disease outcomes, but not the IFN-β knockout mice.[243] However, when another study used blocking of IFN-β and other IFN-α subtypes (α 4, 5, 11 and 13) or combined, it could not reproduce the same results. These differences in the findings could be due to study designs. Further studies on different IFN I subtypes are necessary to uncover the mechanisms behind distinct responses[211,237].

Furthermore, another study showed that LCMV Cl-13 and LCMV-arm elevated the IFN-α expression, but only LCMV Cl-13 expressed high levels of IFN-β.[234] In addition, a study by Ng et al. 2015 showed IFN-β can only be attributed to chronic LCMV-Cl-13 infection. They showed IFN-β is not essential for initial antiviral activities. Blocking IFN-β also improved the splenic organization, T cell-mediated antiviral responses, etc.[81]

2.9.8 IFN I therapies: IFN-α and IFN-β based therapies

IFN responses can be beneficial or detrimental depending on the timing, magnitude source of IFN production and the biological context.[245] Despite its dual nature, IFN-Is continue to play positive roles throughout the infection. However, generalized IFN I therapies may not be efficient for all types of infections and tumour treatments due to their differential responses.

Currently, IFN I cancer therapies have been used for various cancers, but several limitations are linked to both IFN-α or IFN-β based treatments. This section will discuss some evidence of IFN I therapies and approaches used in developing treatments.

IFN-α derived adjuvant therapies are approved for melanoma.[246] IFN-α based therapies have also been used in chronic myeloid leukemia. When IFN-α therapy was used instead of a tyrosine kinase inhibitor imatinib, IFN Is could target a rare Chronic myeloid leukemia (CML) stem cell population following chemotherapy-mediated tumour killing.[211]

IFN-α2b derivatives have been used in the clinic for a long time, but side effects such as toxicity upon persistent or systemic administration are common limitations.[211] Another vital attempt was the RNA-lipoplex vaccine, which could reject various tumours in mouse models.[247] Another successful attempt in cell-based therapies was modifying the human monocyte hematopoietic progenitors. The manipulated monocytes were able to inhibit breast cancer progression in a mice model.[248]

Even though IFN-β based therapies for cancer are fewer compared to IFN-αs, in vitro studies showed that IFN-β has strong potency to induce apoptosis in melanoma compared to IFN-α subtypes.[249] Another study also showed that increased IFN-β mRNA expression level strongly correlates with triple-negative breast cancer.[250] However, lot more to uncover regarding using distinct IFN I subtypes in different cancers.

The other IFN-β derived therapy used IFN-β fused with anti-tumour antibodies to enhance tumour clearance by improving adaptive immune responses.[251]

Furthermore, several successful attempts to use IFN-α to treat viral infections such as HCV and HBV have been reported in the literature.[168] However, in the case of chronic HIV infection, IFN-α treatment did not show persisting benefits in improving disease progression.[252–257] Studies have also shown the inefficacy of IFN-α therapies on humanized mouse models.[258,259] It should be noted that these studies mainly used IFNα2, the frequently used α subtype. However, interestingly, IFN-β and IFNα14 were shown to be potent against HIV.[259] It has been shown that the IFN-β blockade alone showed improvement in LCMV antiviral responses, but not to the extent of IFNARKO, which blocks the entire IFN I Signalling. [260]

IFNAR blocking is another successful approach used against chronic viral infection. Blocking the IFNAR receptor is a frequently used method to ablate IFN I signaling in chronic infection models. For example, chronic viral infection models showed improved viral control upon IFNAR blockade.[234,236] In another model of chronic HIV infection, IFNAR blockade showed improved disease outcomes, especially in conjunction with simultaneous anti-retroviral therapy.[261] Another study also showed the effectiveness of IFNAR blockade and chimeric antigen receptors therapy (CART) therapy in chronic HIV infection in a humanized mice model. [262]

Moreover, ***how could these differential outcomes arise from the shared receptor-mediated Signalling?*** I will summarize some findings explaining the potential mechanisms behind these various Signalling outcomes.

2.9.9 IFN I responses: Robust and tunable activities and their dual nature

Dual nature of IFN Is

IFN I responses are now implicated in many physiological conditions, including bacterial, parasitic, and fungal infections and in anti-tumour responses. They are also implicated in immune suppression against tumour or viral infection, resulting in adverse outcomes such as chronic infection and tumour progression. [6,170,263–266]

Overacting IFN I responses during acute antiviral response or prolonged activation during chronic viral infection leads to deleterious effects to the host. IFN Is orchestrate antiviral responses in many ways, such as inhibiting viral replication via ISG production or inducing innate and adaptive immune responses to combat the infection. While the antiviral role of IFN Is is well accepted and targeted in many therapies, immune-modulatory and detrimental roles in immune pathologies, including autoimmune diseases, cancers and chronic viral infections, are emerging recently.[182,264]

Chronic viral infections-associated disease outcomes are linked to sustained IFN I signalling, which was illustrated nicely in many studies. For instance, prolonged IFN I Signalling in monkey models of AIDS has been linked to disease progression. [182]

Moreover, IFN Is responses are classified into robust and tunable activities. Robust activities are common to all cell types, can occur even at lower concentrations of IFN Is and are activated even at lower surface receptor levels. This includes the responses to viral and other infectious stimuli, etc. Tunable activities are cell-type specific and only induced at high IFN I concentrations like 1000-fold compared to robust activities. In addition, tunable activities are activated by IFN-β, not

IFN-α subtypes and sustained long after the induction.[267,268] They include cytokines and chemokines activities, inflammation, taxis, antiproliferative and immunomodulatory activities. [267] Thus, this variability in IFN I signalling outcomes likely depends on the surface receptor abundance, differential binding affinities of IFNs, other regulatory mechanisms, the context of signalling, crosstalk of different signalling pathways, post-translational modifications etc. Furthermore, the cell type specificity, ISG expression patterns and their kinetics can also contribute to this variability.[169,172,182]

Multiple simultaneous counter-regulatory mechanisms are present to balance the IFN I responses. For example, negative feedback mechanisms, including induction of immune inhibitory molecules such as PDL-1, ndoleamine-pyrrole 2,3-dioxygenase (IDO), Ubiquitin Specific Peptidase 18 (USP18), Suppressor of cytokine signaling 1 (SOCS1) and IL-10 servers to modulate the immune response to suppress excessive stimulation.[269,270]

2.9.9.1 Feedback mechanisms and Feedback loop sensitivity

One of the regulatory mechanisms of IFN I signalling is the endocytosis of IFNAR receptors following IFN I binding. Ternary complex formation and ubiquitination upon IFN I binding to IFNAR1 expose the endocytic motifs of the receptors.[271,272] Receptor abundance has been linked to the receptors' endocytosis level. Rapid downregulation of IFNAR receptors following IFN I binding mediates desensitization of cells from further stimulation.[182,273,274]

Other regulatory mechanisms involve negative regulators such as USP18, SOCS1, etc. USP18 is shown to negatively regulate IFN I response by binding to IFNR2 and STAT2 [275] and interfering with the recruitment of IFNAR1 for signaling ternary complex. [276,277]

Higher concentrations of USP18 remain after following long-term induction of IFN Is. Interestingly, IFN-β can efficiently recruit IFNAR1 to form a ternary complex at high

concentration of USP18, but the responsiveness to IFN-α is reduced. This indicates prolonged USP18 levels prevent IFN-α Signalling, but not IFN-β. [172,276,277]

Furthermore, one of the negative regulators of IFN I signalling is Protein kinase D2 (PDK2), which phosphorylates IFNAR1, leading to receptor endocytosis. Endosomes formed by IFN-β induced ternary complex are long-lived and continue to signal through the trafficking process until it fuses with a lysosome. On the other hand, endosomes with IFN-α are short-lived and are recycled back to the cell surface.[278–281] This indicates the differential feedback sensitivities in the IFN I subtype signalling.

Furthermore, the differential expression of IFN I subtypes is another possible mechanism.

Except for IFN-ε which is hormonally regulated, all the other IFN I subtypes depend on their upstream signalling pathways and activated transcription factors such as IRFs for their regulation. Especially, IRF3 and IRF7 are critical in IFN I expression. IRF3 is constitutively expressed in most cell types and IRF7 is expressed and activated downstream of IFN I signalling to amplify IFN I production and ISGs.[208]

In mice, IRF3 can transcribe (Murine IFN) MuIFN-β and MuIFN-α4 in the early phase of the response and the other IFN-α subtypes depend on IRF3 and IRF7. Similarly, HuIFN-β and HuIFN-α1 depend on IRF3 in the early responses and the other HuIFN-α genes depend on IRF3 and IRF7. Thus the IRF3/IRF7 ratio can also modulate the type-I IFN responses during infection. [211]

Moreover, IFN-β promoter region consists of additional responsive elements that bind several other transcription factors such as activating transcription factor-2 (ATF-2), c-Jun and NF-kB via NF-kB signalling, allowing them to express basal IFN levels independent of IRF3. This may be necessary for properly activating the innate immune system during infection.[282–290]

IFN-ε and IFN-κ are out of these discussions as they are more specific for cell types and further characterization of their signalling is still highly warranted. For example, IFN-κ is named after its prevalence in keratinocytes. IFN-ε is hormonally regulated, not via PRR Signalling or IRFs.[202,204–206,291,292]

2.9.9.2 Differential binding affinity

Despite the pleiotropism and redundancy of IFN-1s, recent evidence appreciates IFN I subtypes' unique features in binding affinities. It is now accepted that IFN subtypes display distinct potencies of their shared and unique functions. [293]

All IFN I subtypes bind and signal through the same receptor molecules that consist of two subunits that are IFNAR1 an IFNAR2. Out of these two receptors, IFNAR2 is the high-affinity binding receptor that binds the ligand first and subsequently recruits the low-affinity IFNAR1 to form the ternary complex that initiates the signalling. Generally, IFNAR2 has around nanomolar affinity and IFNAR1 has about micromolar affinity.[294]

Human IFN-β has the picomolar and nanomolar affinity for IFNAR 2 and IFNAR1, respectively. Similarly, IFN-α2 has nanomolar and micromolar affinity for IFNAR 2 and IFNAR1, respectively. Due to its high affinity, IFN-β is capable of signalling independently of IFNAR1, unlike IFN-α.[268,295,296]

Engineered IFN Is can gain IFN-β like properties.[297,298] IFN-ε and IFN-κ are shown to have weak affinities to IFNAR, about 1000-fold lower than IFN-α2. However, the binding affinities were comparable to other IFN-αs.[299] This study showed that human IFN-ε and IFN-κ showed weak potencies to pox virus infection compared to other IFN Is.[299]

3 CHAPTER 3: MODERATE EXERCISE PROMOTES TRAINED IMMUNITY IN MACROPHAGES.

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Keywords: Inflammation; Immunometabolism; Epigenetic modification; oxygen consumption rate; Proton efflux rate; Macrophage polarization; Bone marrow derived macrophages

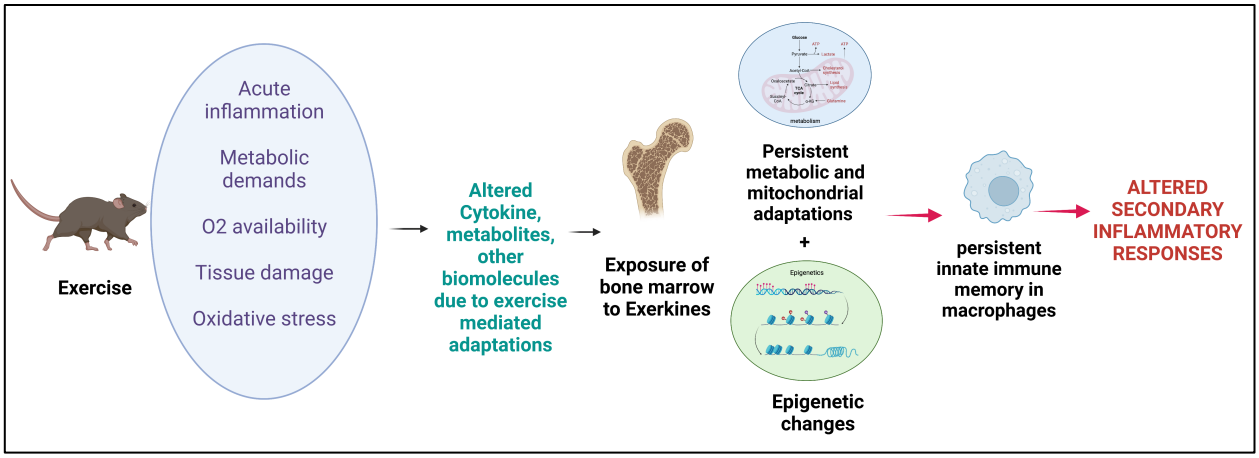
Running title: balancing inflammatory responses by exercise

Funding: This work was supported by the Canadian Institutes for Health and Research.

Data Availability: Data are available upon request from the authors.

This manuscript has been submitted to PLOS biology (March 2023)

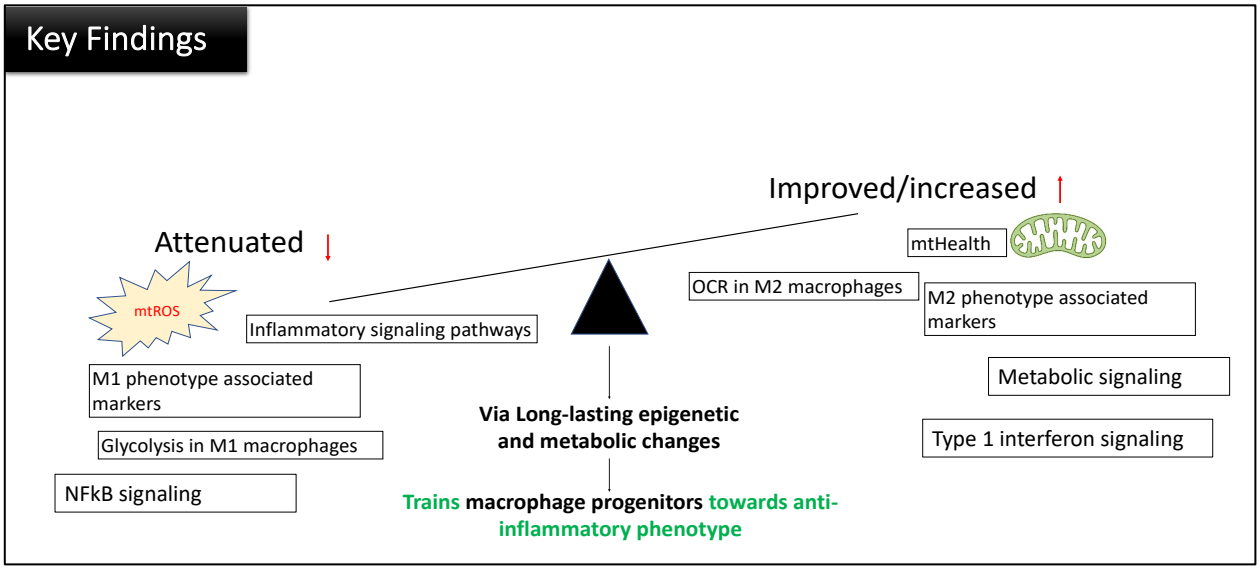
Graphical Abstract



3.1 Abstract

Despite its importance in protecting the host from infections and injury, excessive inflammation may lead to serious human diseases, including autoimmune disorders, cardiovascular diseases, diabetes, and cancer. Exercise is a known immunomodulator; however, whether exercise causes long term changes in inflammatory responses and how these changes occur are still unclear. Here, we show that chronic moderate intensity training of mice leads to persistent metabolic rewiring and changes to chromatin accessibility in bone marrow derived macrophages (BMDMs), which, in turn, tempers their inflammatory responses. We show that BMDMs from exercised mice exhibited a decrease in lipopolysaccharide (LPS) induced NF-κB activation and pro-inflammatory gene expression along with an increase in M2-like associated genes when compared to BMDMs from sedentary mice. This was due to improved mitochondrial quality, increased reliance on oxidative phosphorylation, and reduced mitochondrial ROS production. Mechanistically, ATAC-seq analysis showed changes in chromatin accessibility of genes associated with inflammatory and metabolic pathways. Overall, our data suggest that chronic moderate exercise can influence the

inflammatory responses of macrophages by reprogramming their metabolic and epigenetic landscape.



3.2 Introduction

Inflammation is an essential early immune response to infection and tissue damage and critical for restoring tissue homeostasis [300]. However, a balanced inflammatory response is critical to avoid excessive or prolonged inflammation, which can result in serious diseases such as diabetes mellitus, cardiovascular diseases, cancer, autoimmune and neurodegenerative conditions. In addition, dysregulated systemic chronic inflammation can impair the ability of the host to properly respond to infections, tumours and vaccinations [301–308].

Exercise has long been known to exhibit immunomodulatory effects [309,107,310–317,12,318–320]. Moderate-intensity exercise has been shown to augment immunosurveillance and exert anti-inflammatory effects, thereby providing numerous health benefits, like reducing chronic inflammatory diseases [85,309,321,322]. Decreased visceral fat mass, increased anti-inflammatory myokines in muscles [323,324], reduced expression of TLRs in macrophages and monocytes [325],

attenuated macrophage polarization and infiltration to adipose tissue in mice [326], increased T-regulatory cells, and decreased pro-inflammatory monocytes in human peripheral blood [327,328], have all been suggested as possible mechanisms for the anti-inflammatory effects of exercise. However, how exercise induces these adaptions and changes in inflammatory cells remains unknown.

Importantly, whether exercise alters the immunometabolism of macrophages are largely unexplored [322]. Macrophages are highly plastic and heterogeneous cells that populate most tissues. They play vital roles in tissue homeostasis, activation of inflammatory responses, and resolution of inflammation and tissue remodelling [41]. In fact, macrophages adopt a functional and phenotypic spectrum, which is influenced by their tissue microenvironment [329]. Thus, exercise may be a powerful tool that can be employed to influence the immunometabolism and polarization states of macrophages. In this study, we explored whether moderate exercise could cause persistent adaptation in macrophages, resulting in trained immunity, which is known to occur in innate immune cells due to long-term metabolic and epigenetic changes. To this end, we employed flow cytometry to measure metabolic signaling, mitochondrial oxidative stress and membrane potential and quality, extracellular flux analysis to measure cellular respiration and genome-wide chromatin accessibility assay to evaluate adaptations in bone marrow derived macrophages as possible mechanisms for exercise induced anti-inflammatory effects.

3.3 Objectives and Research questions

We hypothesize that structured exercise programs cause persistent epigenetic and metabolic changes in macrophages, which could provide beneficial effects.

1. Does chronic moderate exercise cause persistent adaptations in Bone marrow-derived macrophages (BMDMs)?

2. What are the molecular mechanisms behind these adaptations?
3. How long do these changes persist?

3.4 METHODS

3.4.1 Exercise training protocol

Six-week-old female C57BL/6J mice were subjected to exercise training on a treadmill. After acclimatization to the treadmill for 5 days (Schneider-Electric) at 10 m/min for the first 5 min and 20 m/min for the next 5 min (total 10 min) at 0° inclination, mice were separated into exercise or sedentary (no exercise) groups. Mice were then exercised at 20 m/min (0° inclination) for 1 h per day (5 days per week) with a 10% increase in speed every week up to a maximum of 30 m/min and continued for 8 weeks (**Fig. 1A**).

3.4.2 Cell culture and reagents.

Bone marrow derived macrophages (BMDMs) were prepared from the femur and tibia of mice and cultured in S+ media, composed of RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Wisent), 2-Mercapthoethanol (Gibco), 1% L-Glutamine-Pyruvate-Penicillin-Streptomycin (Sigma), non-essential amino acids (Gibco) and 25% L929-conditioned media), as previously reported [330]. Lipopolysaccharide (LPS; Escherichia coli; serotype O26:B6), eed blood cell lysis buffer and PBS were obtained from Sigma.

3.4.3 Flow cytometry

For phospho-flow assays, BMDMs were serum-starved for 2 hrs and stimulated with 100 ng/ml LPS in S+ media. Cells were fixed in IC fixation buffer (Thermo-Fisher) at room temperature for 30 min, scraped and transferred to 96 well plates followed by permeabilization with 100% ice cold methanol at 4 ^{o}C for 30 min. Cells were then washed in FACS buffer (PBS containing 2% FBS)

and stained with intracellular phospho-antibody cocktail targeting p-S6 (Alexa Flour 488), p-P38 (PE), and p-Erk1/2 (Alexa Flour 647) (Cell signaling) for 25 min at 4 °C.

For mitochondrial quality assays, BMDMs were induced with LPS (1 µg/ml and 5 µg/ml) for 6 hrs then stained for 10 min at 37 °C either with 2.5 µM MitoSox (Thermo-Fisher) and 40 µM monobromobimane (mBBr; Thermo-Fisher) made in FACS buffer to assess oxidative stress or with 5 µM JC-1 made in FACS buffer (Thermo-Fisher) to assess mitochondrial membrane potential. Similarly, mitochondrial quality measurements were carried out in LPS-treated BMDMs and stained with 50 nM Mitotracker™ Green and Red in FACS buffer [330]. Cells were then analyzed on an Attune NxT flow-cytometer (Thermo-Fisher).

3.4.4 Bioenergetic analyses

Cellular oxygen consumption rate (OCR) and proton efflux rate (PER) were measured using the Seahorse XFe96 Analyzer (Agilent Technologies). 50,000 BMDMs were plated on Seahorse XF plates (Agilent) in S+ media and allowed to adhere overnight. Media was replaced with XF DMEM media pH 7.4 (Agilent) supplemented with glucose, glutamate, and pyruvate then incubated in CO_2 free incubator for at least 1 h prior to loading into the instrument. XFe96 sensor cartridges were hydrated overnight with XF Calibrant at 37°C. Various inhibitors/inducers were loaded to the ports depending on the assay.

For Mitochondrial stress test, Oligomycin (sigma), Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP; sigma), Rotenone (sigma), and Antimycin (sigma) were added sequentially, as per manufacturer's instructions. Absolute oxygen consumption rate in pmol O_2 per minute was calculated using report generator [331–333]. Cell numbers were normalized using the CyQuant (Thermo-Fisher) method.

For real time macrophage polarization, inducers such as LPS (100 ng/ml) + IFNγ (20 ng/ml), or IL4 (20 ng/ml) were injected through port A. PER (pmol H+/min) values were calculated from the real time ECAR (mpH/min) values measured for 6 hrs every five minutes and normalized to basal values prior stimulations.

3.4.5 Quantitative real-time-PCR (Q-PCR) analysis

Total RNA was isolated using Trizol extraction method and reverse transcribed with M-MuLV reverse transcriptase (New England BioLabs), oligo dT primers (Qiagen) and dNTPs (Thermo-Fisher) into cDNA. Genes of interest were amplified using Ssoadvanced SYBR Green Master mix (Bio-Rad) in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Cq values were normalized to RPLP0 control, which was selected among 30 housekeeping genes based on the results of normalization gene array (Bio-Rad).

3.4.6 Immunoblotting

BMDMs were treated with 100 ng/ml LPS, and cell lysates were separated using 10% SDS gels and transferred to polyvinylidene difluoride membranes (PVDF; Bio-Rad) membranes and probed with corresponding antibodies such as total IκB-α (1:1000; Cell Signalling) and Phospho-IRF3 Ser396 (1:1000; Cell Signalling). Blots were imaged in ChemiDoc MP Imaging System (Bio-Rad).

3.4.7 Bulk ATAC-seq library preparation

Samples were prepared as previously described [334]. Briefly 60,000 viable cells per sample were pelleted at 500 RCF at 4°C for 5 min. Supernatant was removed and cells were re-suspended in 50ul of cold resuspension buffer (RSB) containing 0.1% NP40, 0.1% Tween-20 and 0.01% digitonin. Cell suspension was incubated on ice for 3 minutes before being washed out with 1 ml of cold RSB containing 0.1% Tween-20. Cells were mixed by inversion prior to pelleting at 500 RCF at 4°C for 5 min. Supernatant was removed, cells were re-suspended in 50ul of transposition

mix and incubated at 37°C for 1 hour in a thermomixer (Eppendorf) set to 1000 RPM. Transposition mix was purified using the Qiagen MinElute Reaction Cleanup Kit (Qiagen) and eluted in 20ul of water. 1ul of sample was taken into a qPCR reaction to determine the optimal number of PCR cycles required for amplification without reaching saturation and based off the measured cycle number, the remaining 19ul of each sample was amplified. Libraries were purified using AMPure XP beads (Beckman Coulter) using a double-sided bead cleanup protocol set to 0.7x-1.0x. This clean up was performed twice to remove the large molecular fragments.

Purified libraries were evaluated for enrichment by qPCR using primers designed against open regions (KAT6B and GAPDH) compared against closed regions (QML93 and SLC22A3). Samples that had a fold enrichment greater than 10 were sequenced.

The libraries were quantified by qPCR then normalized and pooled to 1.25nM. Each 1.25 nM pool was denatured using 4ul of 0.2N NaOH (Sigma) for 8 minutes at room temperature before being neutralized with 5ul of 400mM Tris-HCl (Sigma). The neutralized pool was loaded immediately onto a NovaSeq 6000 SP flow cell. Samples were sequenced with the following run parameters: read 1 – 50 cycles, read 2 – 50 cycles, index 1 – 8 cycles, index 2 – 0 cycles.

3.4.8 ATAC-seq data analysis

To determine differential chromatin accessibility between samples, occupancy analysis (using DiffBind) was carried out to determine the unique and common peaks between the groups. Diffbind matrix was then analyzed and visualized using DEBrowser, a differential expression analysis and visualization tool [335]. Principal component analysis (PCA) was then applied to cluster samples with trimmed mean of M-values (TMM) method and analyze the top 1000 most variant genes between the compared sample groups. Using DESeq 2 (Love et al. 2014), differential expression (DE) analysis was performed between the sample groups as well. DESeq 2 analysis

provides an output of differentially expressed genes (DEGs) listed by their relative p-adj values and fold change. Positive and negative log-fold change values determined if specific regions were upregulated or downregulated between the compared sample groups.

3.4.9 Statistics

The results are presented as means with standard error of the mean (SEM). Significance is determined by a p-value of less than 0.05. Each experiment was conducted multiple times. N values indicated in the figure captions are biological replicates. When comparing two groups, an unpaired t-test is used. When comparing more than two groups, ANOVAs are conducted and followed by a post-hoc analysis, Tukey test, depending on the type of ANOVA. GraphPad Prism 9.4.1" (San Diego, CA, USA) was used to perform the statistical analysis for all the experiments.

3.5 RESULTS

3.5.1 Moderate exercise differentially affects inflammatory signaling in BMDMs following LPS stimulation

A growing number of studies support the notion that moderate exercise can lower inflammation, while reducing the risk for viral infections, particularly, upper respiratory tract infections [84,85,101,321,336–341]. However, the molecular mechanisms through which exercise mediates these effects remain poorly understood. Moreover, most studies have focused on the transient effects of exercise on immune responses [114,115,317,340–348]. Whether exercise can cause prolonged and persistent effects on inflammatory responses is not well understood. Therefore, we examined how chronic moderate exercise alters the inflammatory and antiviral responses in bone marrow derived macrophages (BMDMs) in mice (**Fig. 3.1A**). Given that BMDMs require 5-7 days to mature in culture, this allowed us to assess the persistent effects of exercise independently from the transient effects seen shortly after an exercise bout. BMDMs obtained from sedentary and exercised mice

were stimulated with LPS to trigger inflammatory (pro-inflammatory cytokines) and antiviral (Type I interferons) responses. Our data show that expression of inflammatory genes (IL-1β, TNFα) was significantly reduced in BMDMs prepared from exercised mice when compared to those prepared from sedentary controls (**Fig. 3.1B-E**). Moreover, anti-inflammatory genes (IL-10, TGFβ) were also reduced in exercised BMDMs, suggesting attenuation of a common upstream signaling pathway (**Fig. 3.1F-I**). Remarkably, expression of interferon-β (IFNβ), a key antiviral cytokine, was markedly higher in exercised BMDMs (**Fig. 3.1J, K**). This may explain how moderate exercise lowers the susceptibility to viral infections (URTI). To understand this dichotomy, we measured NF-κB and IRF3 activation, the two main transcription factors induced following LPS stimulation to induce inflammatory and interferon genes, respectively. NF-κB activation was significantly reduced in BMDMs prepared from exercise mice (**Fig. 3.1L**), while IRF3 activation was markedly higher in exercised BMDMs (**Fig. 3.1M**). Interestingly, IRF-3 and IFNβ are implicated in macrophage polarization toward an anti-inflammatory phenotype [349–351]. Collectively, these results show a persistent anti-inflammatory effect of exercise in bone marrow macrophages. This is consistent with the reports that showed an anti-inflammatory macrophage phenotype conversion in liver [352,353] and adipose tissues [326].

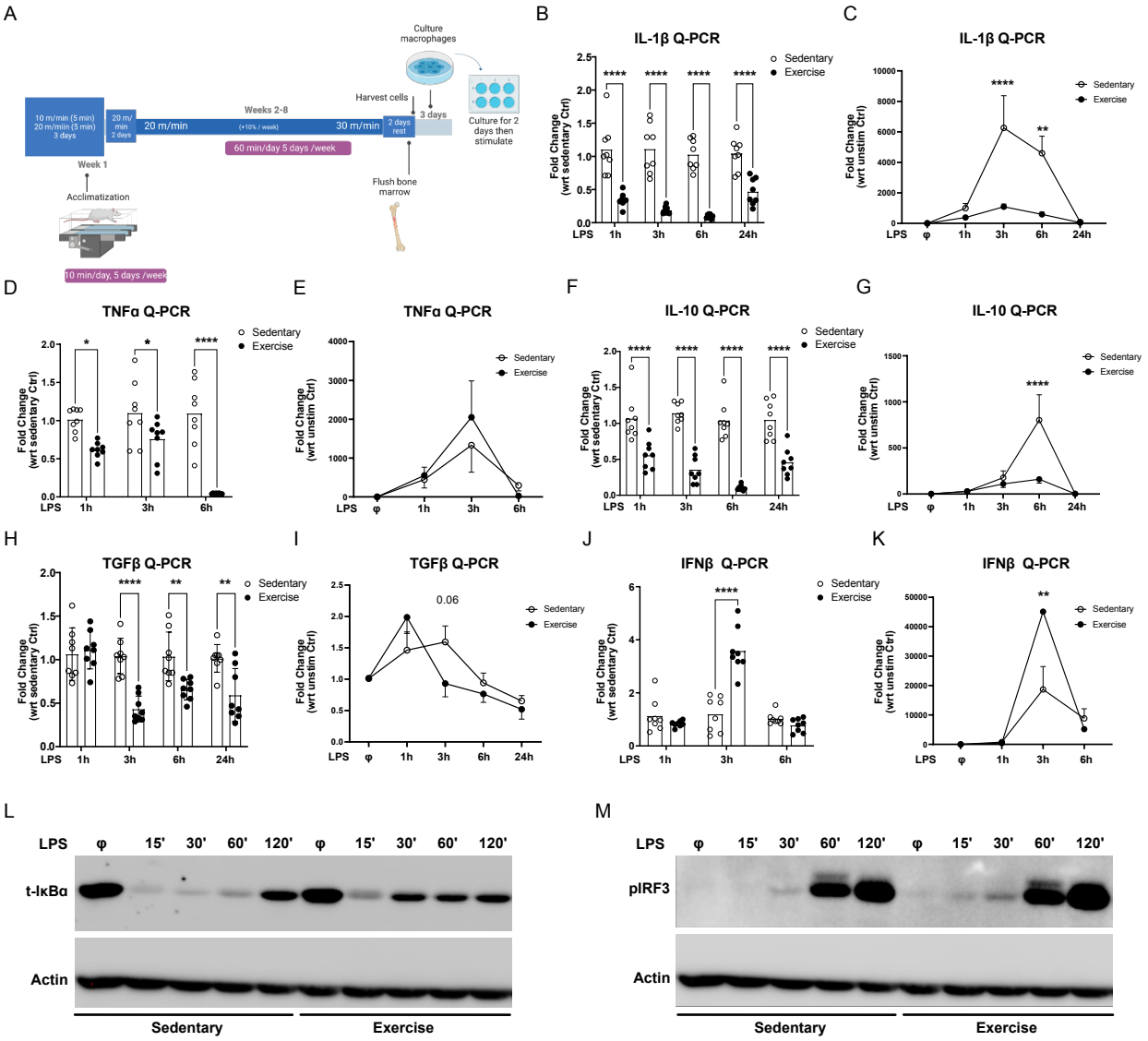


Fig. 3.1: Moderate exercise alters inflammatory and anti-viral responses in BMDMs

BMDMs from Exercised and sedentary C57 BL/6N mice were stimulated with 100 ng/ml LPS for the indicated times. (**A, B**) Gene expression of IL-1β was evaluated by real-time PCR (Q-PCR). Results were normalized to RPLP0 and reported as relative fold change with respect to 1 h treatment (**A**) or sedentary unstimulated control (**B**). Gene expression of TNFα (**C, D**), IL-10 (**F, G**), TGFβ (**H, I**), and IFNβ (**J, K**) were evaluated by Q-PCR as in panel A, B. Each symbol represents an individual mouse (n=8 per group). Data were compared by multiple comparisons from a 2-way anova. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Total IκBα (t- IκBα; **L**) and phospho-IRF3 (pIRF3; **M**) levels were evaluated by Western Blotting in 100 ng/ml LPS treated BMDMs for the indicated time points. Actin served as a loading control. Blots are representative of 3 independent experiments.

3.5.2 Moderate exercise alters expression of genes and metabolic signatures associated with M1 and M2 macrophage phenotypes

Classically activated macrophages (M1-like) upregulate glycolysis and limit oxidative phosphorylation (OXPHOS) in order to promote pro-inflammatory gene expression [354]. They do so by increasing the expression of inducible nitric oxide synthase (iNOS) to inhibit mitochondrial respiration, as well as Hypoxia-inducible factor-1α (HIF1α) and phosphofructokinase (PFK2) to enhance glycolysis [354]. Conversely, alternatively activated macrophages (M2-like), upregulate Heme-oxygenase-1 (HMOX-1), Arginase-1 (ARG1) and Immune-responsive gene 1 (IRG1) to enhance OXPHOS [355–357] in order to promote anti-inflammatory gene expression. Therefore, we asked whether exercise, which is known to alter cellular metabolism in other cells, had an impact on expression of M1-like and M2-like associated genes. Our results show that expression of the M2-associated genes, ARG1 and HMOX-1, were significantly increased 24 h following LPS stimulation in exercised BMDMs compared to sedentary (**Fig. 3.2A, B**). Similarly, expression of IRG1, which encodes an enzyme that catalyzes cis-aconitate conversion into itaconate, an anti-inflammatory molecule required for metabolic reprogramming of macrophages [358–361], was significantly increased 6 h following LPS stimulation in exercised BMDMs (**Fig. 3.2C**). On the other hand, gene expression of the M1-associated genes, uPKF2, HIF1α and iNOS were decreased in macrophages prepared from exercised mice compared to those from sedentary mice (**Fig. 3.2D-F**). These data indicate that moderate exercise may be tilting the M1/M2 balance toward the anti-inflammatory phenotype by altering cellular metabolism.

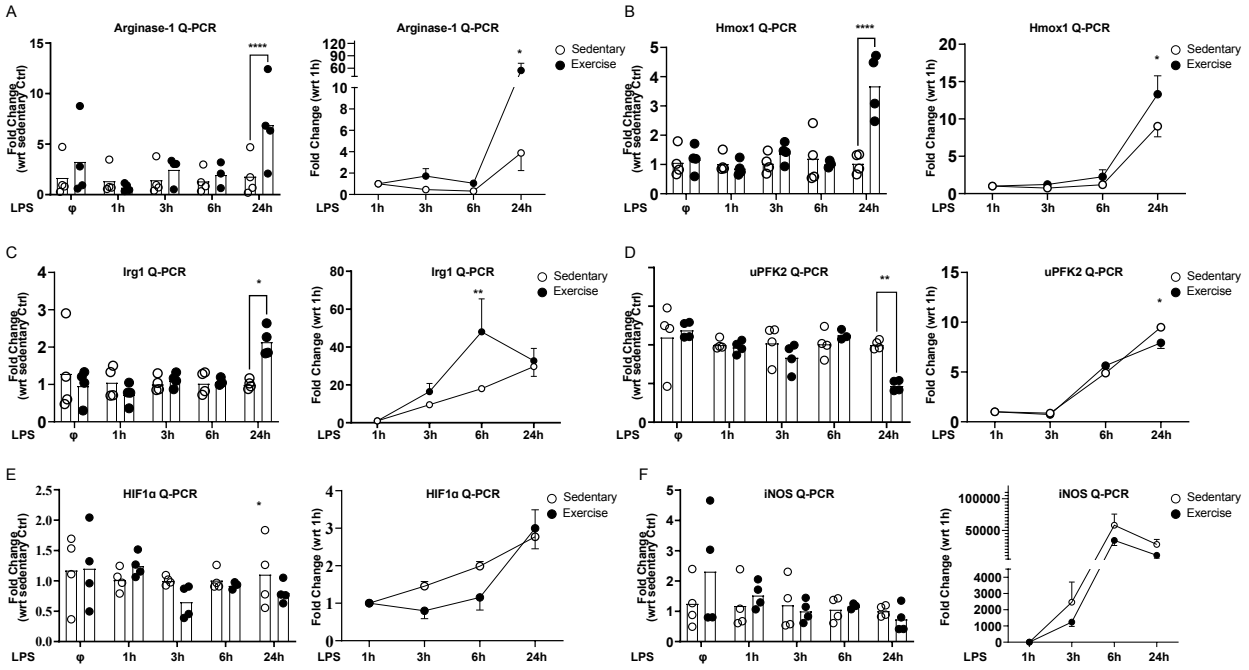


Fig. 3.2: Effect of moderate exercise on M1 and M2-associated genes

BMDMs from Exercised and sedentary C57 BL/6N mice were stimulated with 100 ng/ml LPS for the indicated times and gene expression of Arginase-1 (**A**), Hmox1 (**B**), Irg1 (**C**), uPFK2 (**D**), HIF1α (**E**) and iNOS (**F**) was evaluated by real-time PCR (Q-PCR). Results were normalized to RPLP0 and reported as relative fold change with respect to 1 h treatment. Each symbol represents an individual mouse (n=4 per group). Data were compared by multiple comparisons from a 2-way anova. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

To test this hypothesis, we employed phospho-flow cytometry to measure the activation of three metabolic pathways that have been associated with regulating inflammatory responses [362]. Indeed, LPS stimulated BMDMs from exercised mice exhibited a significant increase in mTOR (p-S6) and MAPK (p-P38 and p-Erk1/2) signaling when compared to BMDMs from sedentary mice (**Fig. 3.3**). Taken together, our results indicate that chronic moderate exercise could cause metabolic rewiring in macrophages and thereby altering their responses to inflammatory stimuli.

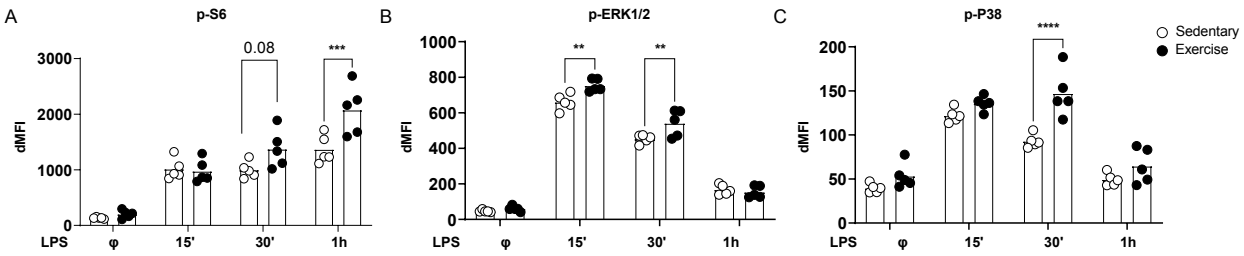


Fig. 3.3: Increased metabolic signaling in BMDMs following moderate exercise
BMDMs from Exercised and sedentary C57 BL/6N mice were stimulated with 100 ng/ml LPS for the indicated times and intracellular levels of phosphorylated signaling proteins, pS6 (**A**), pERK1/2 (**B**) and pP38 (**C**), were evaluated by flow cytometry. Data was plotted as bar graphs of dMFI, which is the mean fluorescence intensity (MFI) of each protein minus that of the fluorescence minus one (FMO) control. Each symbol represents an individual mouse (n=5 per group). Data were compared by multiple comparisons from a 2-way anova. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

3.5.3 Mitochondrial quality is improved in BMDMs following moderate exercise

Mitochondria play a key role in the metabolic reprogramming that controls inflammation and macrophage activation [363–365]. Thus, mitochondrial (mt) quality and functional parameters such as ROS production and membrane potential have a significant impact on the metabolic and inflammatory state of the cells. Therefore, we investigated how moderate exercise affects mitochondrial quality and overall oxidative state of BMDMs. There was a significant reduction in mt ROS production at baseline and following LPS stimulation in BMDMs prepared from exercised mice compared to those from sedentary mice (**Fig. 3.4A-C**). This was accompanied by an increase in reduced glutathione levels in exercised BMDMs (**Fig. 3.4A, B**). Importantly, mt membrane potential was higher in exercised BMDMs at baseline and following LPS stimulation, when compared to sedentary BMDMs (**Fig. 3.4D, E**). These data, combined with an increase in functional mitochondria (**Fig. 3.4F, G**) in exercised BMDMs, show that moderate exercise can improve overall mitochondrial quality in BMDMs, which is reminiscent of the mitochondrial adaptation seen in muscle cells following exercise. Furthermore, these adaptations might explain the remodelling of metabolic pathways we observed in BMDMs following moderate exercise.

M. Thasan et al. Figure 4

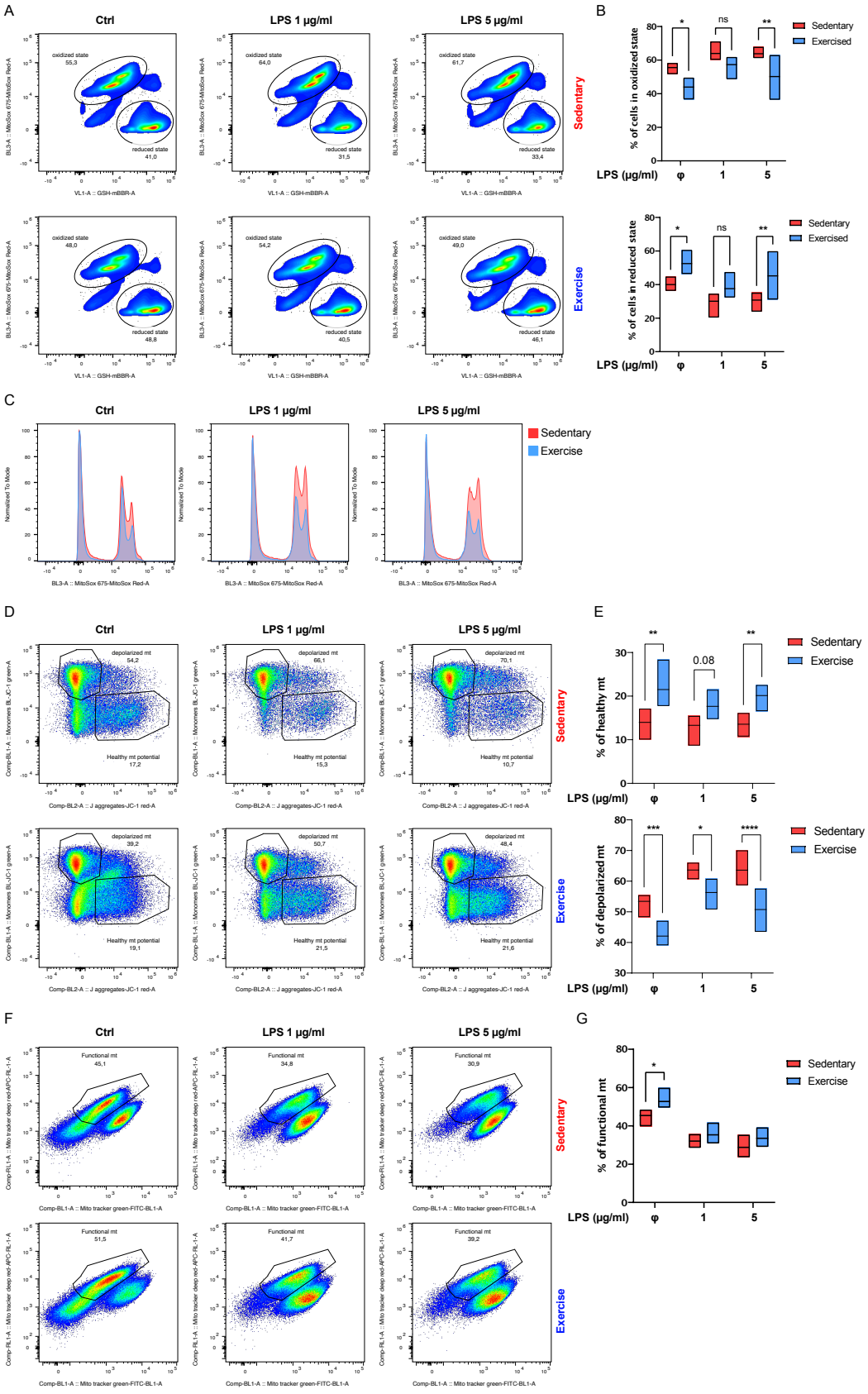


Fig. 3.4: Enhanced mitochondrial function and quality in BMDMs following moderate exercise. (**A-C**) BMDMs from exercised and sedentary mice were stimulated or not with 1 μg/ml and 5 μg/ml LPS for 6 hours followed by flow cytometric measurements of mtROS (mitoSox) and intracellular GSH levels (GSH-mBBr). (**A**) Representative plots showing percentages of cell populations in oxidized (MitoSox high, GSH-mBBr low) and reduced (MitoSox low, GSH-mBBr high) state. (**B**) Box plots showing the distribution of the data representing the percentages of cell populations in oxidized (top) and reduced (bottom) state, as in A (n=5 per group). (**C**) Representative histograms showing an overlay of mtROS measurements from sedentary and exercised BMDMs, as in panel A. (**D-E**) BMDMs from exercised and sedentary mice were stimulated as in panels A-C and stained with JC-1 dye to assess mitochondrial membrane potential (**D**). Representative plots showing percentages of cell populations with depolarized (JC-1 monomer high, aggregate low) and healthy (JC-1 monomer low, aggregate high) mt membrane. (**E**) Box plots showing the distribution of the data representing the percentage of cells with healthy (top) and depolarized (bottom) mitochondria, as in D (n=5 per group). (**F-G**) BMDMs from exercised and sedentary mice were stimulated as in panels A-C and stained with Mitotracker green and deep red dyes to assess mitochondrial quality (**F**) Representative plots showing percentages of cell populations with functional mitochondria (Mitotracker deep red high, Mitotracker green high). (**G**) Box plots showing the distribution of the data representing the percentage of cells with functional mitochondria, as in F (n=5 per group). Data were compared by multiple comparisons from a 2-way anova. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

3.5.4 Moderate exercise enhances mitochondrial function in BMDMs

A plethora of research now shows that activation and polarization of macrophages is closely linked to the way they metabolize glucose to produce ATP via glycolysis and OXPHOS [366–369]. Using extracellular flux analysis, we measured oxidative and glycolytic metabolism of naive, M1 and M2 polarized BMDMs to examine whether the mitochondrial adaptations following exercise are associated with metabolic reprogramming in the cells. Oxygen consumption rate (OCR) and proton efflux rate (PER) were analyzed as surrogates for oxidative phosphorylation and glycolysis, respectively. BMDMs from exercised mice showed a significantly higher shift in maximal respiratory capacity and spare respiratory capacity OCR values upon M2 polarization compared to the sedentary group (**Fig. 3.5A, B, E**). Basal respiration and non-mitochondrial respiration were not changed between the two groups (**Fig. 3.5C-E**). Conversely, when measuring glycolytic rate, as represented by PER, in real-time following M1 polarization, BMDMs exhibited a significantly

smaller increase in glycolytic rate compared to sedentary counterparts (**Fig. 3.5F-G**). Taken together, these findings indicate that exercise can induce metabolic and functional reprogramming in BMDMs.

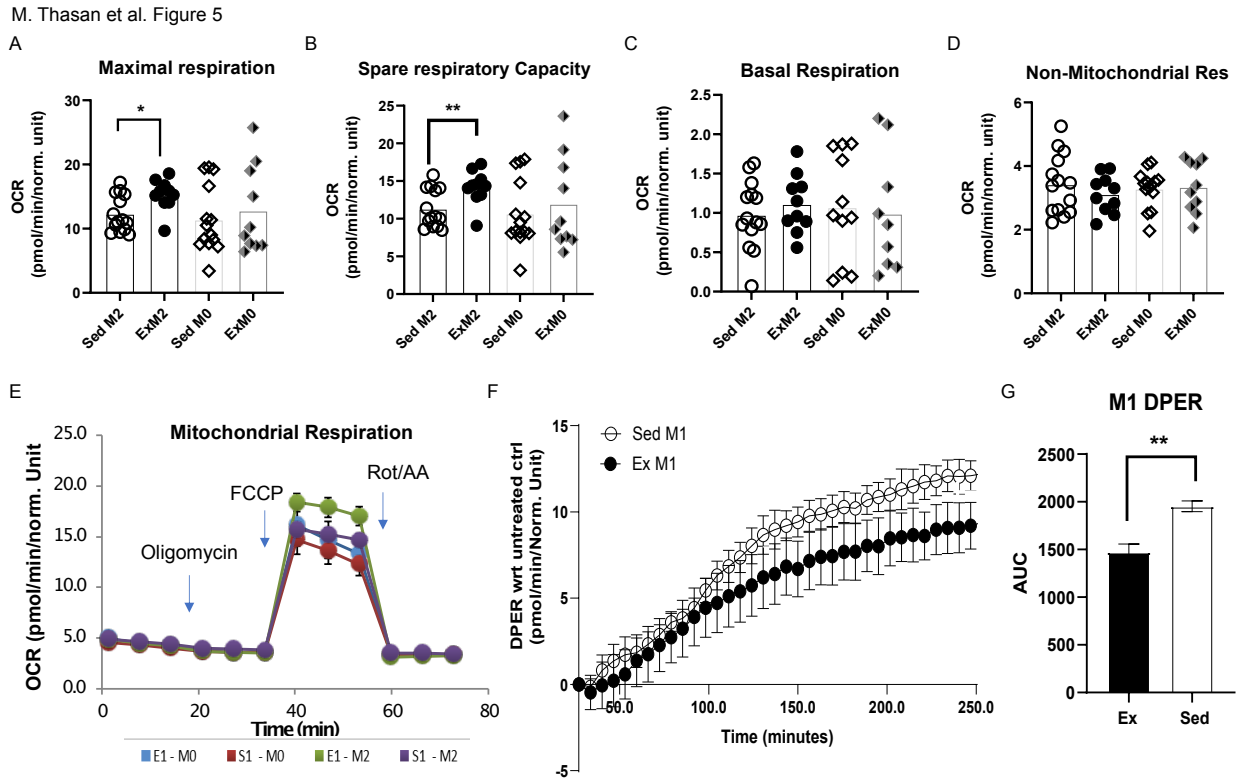


Fig. 3.5: Exercise increases oxygen consumption in M2 polarized BMDMs while reducing glycolysis in M1 polarized BMDMs

BMDMs from exercised and sedentary mice were polarized to M1 and M2 macrophages by incubation in media containing 100 ng/ml LPS + 20 ng/ml IFN-γ (M1) and 20 ng/ml IL4 (M2) for 24hrs. (**A-E**) OCR was assessed using mito stress assay. Graphs representing the Maximal respiration capacity (**A**), Spare respiratory capacity (**B**), Basal respiration (**C**), and Non-Mitochondrial respiration (**D**). Each measurement represents the average of readings of 7 sedentary and 5 exercised mice. Cell numbers were normalized using Cy-Quant method. (**F**) BMDMs were polarized into M1 macrophages at t=0 and proton efflux rate (PER) was measured over time to assess changes in glycolytic rate as the cells were being polarized in real time. Data represents mean DPER ±SEM, metabolic shift upon M1 polarization (PER M1- PER M0) (**G**) Area under the curve (AUC) of the line graphs in panel F. Data was normalized to baseline. Data were compared with a nonparametric student t-test. ** $p<0.01$.

3.5.5 Exercise mediated adaptions in BMDMs begin to wane two weeks after cessation of training

To test how long the metabolic and functional adaptations in BMDMs persist in exercised mice after cessation of training, we prepared BMDMs from mice that have undergone the same chronic moderate exercise training program with or without a two-week resting period. Sedentary mice served as a control group. We then compared metabolic signaling and mitochondrial quality and function across all three groups. Oxidative stress and mitochondrial potential were reduced to the sedentary level when exercise was stopped for two weeks (**Fig. 3.6A-C**). This may be due to the dynamic nature of mitochondrial turnover. Surprisingly, the metabolic switch indicated by reduced PER following M1 polarization persisted even after the two-week rest period (**Fig. 3.6D, E**). Furthermore, the enhancement in metabolic signaling was largely normalized following cessation of training (**Fig. 3.6F-G**).

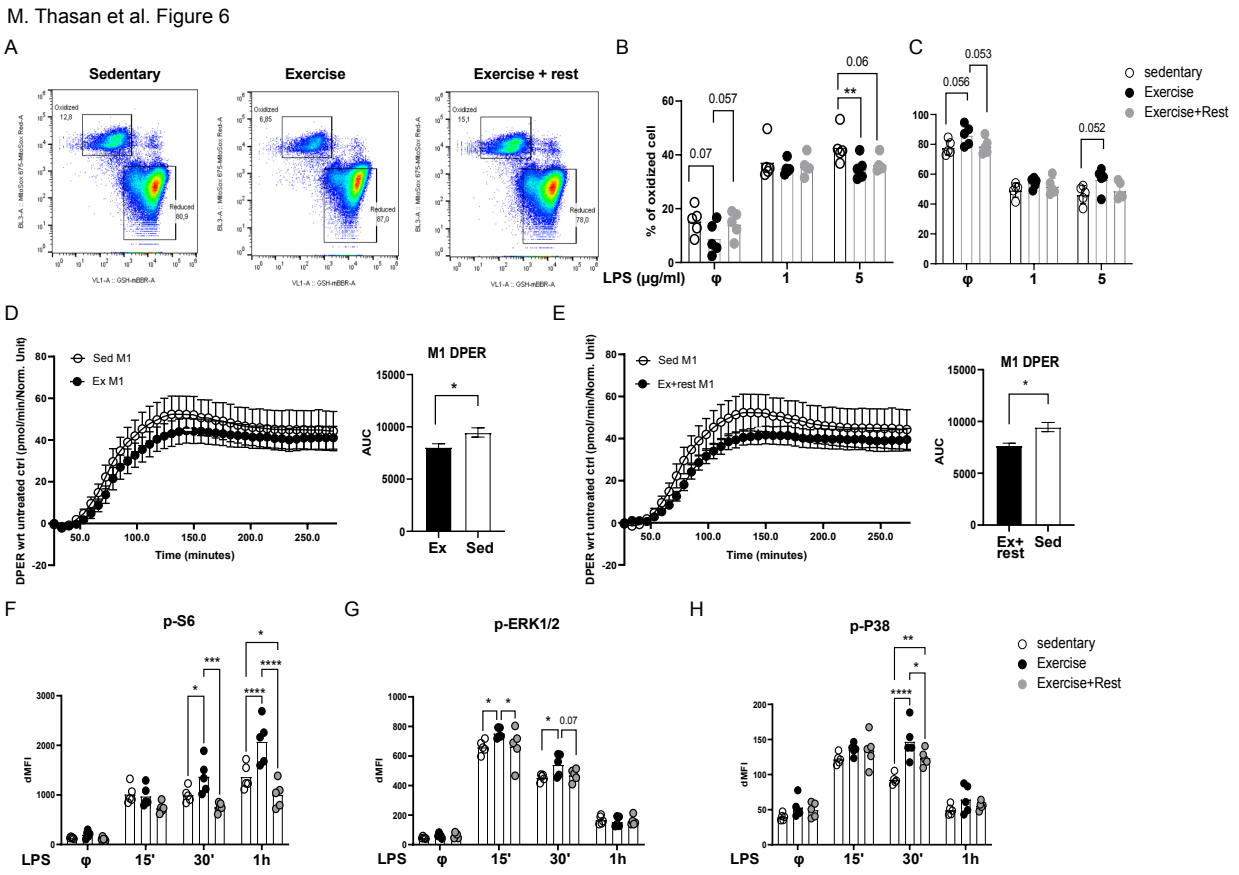


Fig. 3.6: Exercise induced adaptations in BMDMs begin to wane two weeks after cessation of exercise (**A-C**) BMDMs from sedentary, exercised and exercised-rested mice were stimulated or not with 1 μg/ml or 5 μg/ml LPS for 6 hours followed by flow cytometric measurements of mtROS (mitoSox) and intracellular GSH levels (GSH-mBBr). (**A**) Representative plots showing percentages of cell populations in oxidized (MitoSox high, GSH-mBBr low) and reduced (MitoSox low, GSH-mBBr high) state. Box plots showing the distribution of the data representing the percentages of cell populations in oxidized (**B**) and reduced (**C**) state, as in A (n=5 per group). (**D, E**) BMDMs were polarized into M1 macrophages (100 ng/ml LPS, 20 ng/ml IFN-γ) at t=0 and proton efflux rate (PER) was measured over time to assess changes in glycolytic rate as the cells were being polarized in real time to compare sedentary to exercised (**D**) and exercised-rested (**E**) groups. Area under the curve (Right panel) of the line graphs in panels D and E. Data was normalized to baseline. Data were compared with a nonparametric student t-test. * $p<0.05$. (**F-H**) BMDMs were stimulated with 100 ng/ml LPS for the indicated times and intracellular levels of phosphorylated signaling proteins, pS6 (**F**), pERK1/2 (**G**) and pP38 (**H**), were evaluated by flow cytometry. Data was plotted as bar graphs of dMFI, which is the mean fluorescence intensity (MFI) of each protein minus that of the fluorescence minus one (FMO) control. Each symbol represents an individual mouse (n=5 per group). Data were compared by multiple comparisons from a 2-way anova. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

3.5.6 Moderate exercise induces trained immunity in BMDMs

Given the persistent effects imposed by moderate exercise on inflammatory signaling, metabolism and mitochondrial function, we hypothesized that moderate exercise may be inducing cryptic long-term epigenetic changes in BMDMs without overt transcriptional changes [148]. Therefore, we analyzed global changes in chromatin accessibility in unstimulated BMDMs obtained from sedentary or moderately exercised mice by ATAC-seq (Assay for Transposase-Accessible Chromatin). Principal-component analyses (PCA) of ATAC-seq peaks showed a clear difference in clustering of sedentary (S) compared to exercise (E) samples (**Fig. 3.7A**). Rigorous statistical analysis revealed significant changes in chromatin accessibility with 130 increased and 176 decreased open chromatin regions (OCRs) (**Fig. 3.7B, C; Table S1**). We then mapped OCRs that were increased in the exercise group to nearby genes (**Fig. 3.7D**). Intriguingly, GO analysis of genes associated with exercise-induced OCRs showed processes involved in regulation of metabolic activity, cytokine production, and immune cell activation (**Fig. 3.7E; Table S2**). Next, we employed motif enrichment analysis to investigate the enriched ATAC-seq peaks in exercised BMDMs. Remarkably, we observed an enrichment in SPI1/PU.1, a transcription factor essential for macrophage differentiation [370], as well as other transcription factors involved in regulating macrophage function and polarization, including cEBPβ, NFATc1 and RUNX2 (**Fig. 3.7F**). Given its key role in promoting M2-like macrophage polarization and associated genes [371], we examined the differential ATAC-seq peaks centred on the cEBPβ motif, which was significantly enriched in BMDMs from exercised mice (**Fig. 3.7G**). We found that the regulatory regions of M2-associated genes (CD206, MerTk and Maml2) that contained cEBPβ binding sites were more accessible in BMDMs from exercised mice compared to those from sedentary (**Fig. 3.7H-J**). Contrastingly, M1-associated, and inflammatory genes (Src, MMP12 and Hoxa13/Hottip) were less accessible in

exercised BMDMs (**Fig. 3.7K-M**). Altogether, these results show that exercise can induce long-term adaptations in BMDMs by increasing chromatin accessibility of certain M2-associated transcription factors and genes, while reducing accessibility of M1-associated genes.

M. Thasan et al. Figure 7

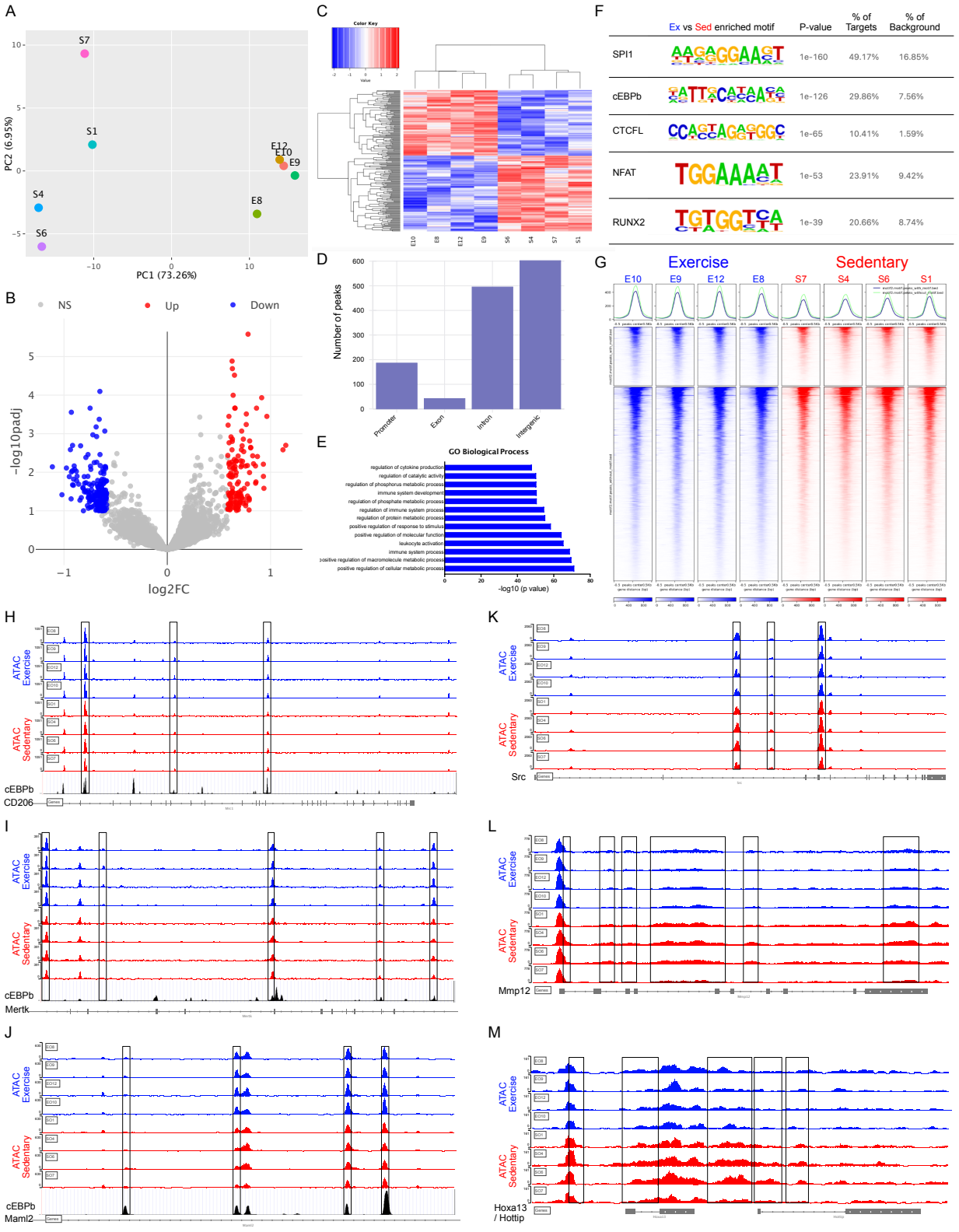


Fig. 7: (**A**) PCA plot of ATAC-seq data showing clustering of changes in chromatin accessibility between exercised and sedentary BMDMs. (**B**) Volcano plot showing open chromatin regions (OCRs) that were increased (red) or decreased (blue) in exercise versus sedentary BMDMs. (**C**) Heatmap of ATAC-seq signal representing the increased and decreased OCRs as in panel B. (**D**) Representation of the distribution of the OCRs that were increased in exercised BMDMs. (**E**) Top GO terms for Biological process of the genes where increased OCRs were mapped to. (**F**) Top ranking motifs that were enriched in exercised BMDMs identified by Homer de novo motif analysis. (**G**) Density heatmap of ATAC-seq signal, centered at the cEBPβ motif and ranked by accessibility comparing sedentary and exercised BMDMs. (**H-M**) Genome browser visualizations of four biological replicates of exercise and sedentary BMDMs, each, showing ATAC-seq (normalized) signal tracks of CD206 (**H**), Mertk (**I**), Maml2 (**J**), Src (**K**), Mmp12 (**L**), and Hoxa/Hottip (**M**). Opening/closing sites are highlighted with black boxes.

3.6 Discussion

It has been well documented that moderate exercise can induce systemic and tissue level anti-inflammatory effects and thereby provide numerous health benefits regarding chronic diseases and infections.[17,107,314,315,317,320–322,325,372–376]. This study demonstrates that chronic moderate exercise can do so by promoting trained immunity in bone marrow derived macrophages (BMDMs) and enhancing their mitochondrial quality and function. Trained immunity is a concept adapted recently and occurs due to functional reprogramming of innate immune cells via metabolic and epigenetic reprogramming [147,148,377]. Indeed, we show via a genome-wide ATAC-seq approach that exercise increased chromatin accessibility in regions associated with M2-like anti-inflammatory genes and reduced accessibility of those associated with M1-like pro-inflammatory genes.

In line with previous studies [325,353,373], we observed a reduction in inflammatory cytokines, such as IL1-β and TNF-α, following moderate exercise training. However, contrary to a study in mouse splenocytes that showed no effects of exercise on the anti-inflammatory cytokines, IL-10 and TGF-β [327], we found that gene expression of IL-10 and TGF-β were attenuated following chronic moderate exercise in BMDMs. These observations suggest that NFκB activation, which controls

the expression of the aforementioned pro- and anti- inflammatory cytokines, is attenuated following moderate exercise training.

There is a paucity of research that explores the molecular mechanisms of how exercise affects inflammatory responses. NF-κB and IRF3 are the two principal transcription factors that drive gene expression of inflammatory cytokines and type-I interferons, respectively. LPS activation of TLR4 has the unique capacity of robustly activating these two signaling pathways. Remarkably, in this study, we show that moderate exercise differentially affects these pathways following LPS stimulation by reducing NF-κB while enhancing IRF3 activation in BMDMs. This may present an important advantage in that moderate exercise can lower the risk of tissue damage imposed by NF-κB driven inflammation, while enhancing antiviral responses driven by IRF3 induced IFN-Is. Importantly, IRF3 and IFN-β are implicated in conversion of macrophages into the anti-inflammatory phenotype [349–351]. This study offered additional insights into signaling pathways that govern immunometabolism and macrophage polarization, where we show that p-P38, p-S6, and p-Erk were elevated in exercise samples. P38 activation has been implicated in Hmox-1 induction and in promoting other macrophage anti-inflammatory phenotype markers [378,379]. Moreover, inhibition of mTOR by rapamycin promotes the production of proinflammatory cytokines [380–382]. Taken together, these observations may imply a metabolic switch that drives BMDMs towards an anti-inflammatory phenotype.

Exercise causes systemic changes in metabolites, proteins, lipids, and inflammatory mediators, which circulate to reach lymphoid tissues, such as the bone marrow. There, these mediators can contribute to metabolic reprogramming and alter mitochondrial quality and function in resident immune cells. Indeed, we observed improved mitochondrial quality and reduced oxidative stress in exercised BMDMs. Mitochondria, now well known as an immune organelle, is a key regulator

of immune signaling [383–387]. Thus, mitochondrial adaptations could also play roles in the metabolic reprogramming and phenotype conversion of BMDMs following exercise. Furthermore, reduction in glycolytic switch upon M1 polarization and enhancement in OCR upon M2 polarization in BMDMs imply a metabolic switch in BMDMs following exercise. Downregulation of uPKF2, a key glycolytic enzyme, also aligns with the above observation. Interestingly, studies have shown that exercise can increase the production of itaconate, a tricarboxylic acid (TCA) cycle metabolite known for its anti-inflammatory properties [353,388–390]. Long-term exposure to such metabolites may contribute to exercise-induced metabolic reprogramming in BMDMs. In fact, our data show that gene expression of Irg1, the enzyme that catalyzes itaconate synthesis from cis-aconitate, is induced in exercised BMDMs.

Trained immunity refers to the functional adaptations of innate immune cells, like monocytes and macrophages, is in response a primary stimulus such as infection or tissue injury. This is achieved through long-lasting epigenetic and metabolic reprogramming, which result in more effective immune responses. In response to subsequent (i.e. secondary) stimuli, trained immune cells can exhibit enhanced immunity or tolerance, a process that dampens the immune system response to protect organs from failure and maintain tissue homeostasis [146–148]. Seeing that moderate exercise has caused metabolic and functional adaptation in BMDMs in response to LPS stimulation, we hypothesized that exercise is acting as the primary stimulus to impose long-term epigenetic remodelling in BMDMs. Therefore, we employed an unbiased approach to evaluate genome wide chromatin accessibility in BMDMs following exercise. Indeed, we observed significant changes in open chromatin regions (OCRs), most of which are associated with cellular metabolism, immune function, and macrophage polarization. Binding of two critical transcription factors (Spi1 and cEBPβ) that regulate M2-like macrophage polarization were enriched following exercise. Spi1

is critical for maintaining CD163+/CD206+ M2-like macrophages in the muscles of aging mice, and for expression of M2-associated markers, including CD206 [370]. cEBPβ, a member of the C/EBP family of proteins, is transcriptionally induced by the CREB transcriptional activator [391] and plays a crucial role in the antibacterial activity of macrophages [392]. When macrophages are activated, CREB-mediated induction of cEBPβ is required for certain M2-associated genes, like ARG1 [371]. Remarkably, we found that chromatin accessibility of the M2-associated genes, CD206 and Mertk, was increased in BMDMs obtained from exercised mice. Moreover, we observed an increase in chromatin accessibility of Mastermind-like (MAML2), which was previously shown to be critical for development of M2-like macrophage [393]. Interestingly, p38 MAPK, which we also showed was increased following exercise, synergizes with IFN-induced JAK-STAT pathways to activate CREB to enhance expression of interferon stimulated genes [394]. This may explain our finding that exercise promoted M2-associated gene expression as well as enhanced expression of IFN I.

On the other hand, we observed a reduction in chromatin accessibility of genes associated with inflammatory and M1-like phenotype. These included MMP12, Hoxa13/Hottip and Src. The M1-associated gene, MMP-12, is not only known as a pro-inflammatory marker [395–397] but also functions as a pro-inflammatory protease [398]. The long noncoding RNA (lncRNA) HOXA transcript at the distal tip (HOTTIP) is upregulated in M1-derived exosomes, and was shown to activate the TLR5/NF-κB signaling pathway [399]. Moreover, HoxA13 can stimulate IL-1bβ secretion in Myometrial Cells [400]. Finally, the protein tyrosine kinase, Src, was implicated in production of pro-inflammatory cytokines, such as TNF-α and IL-6 [401]. Src forms a complex with cIAPs, SHP-1, MyD88, and TRAF1/2 to that is crucial for LPS-induced NF-κB activation and IL-27 production in human monocyte-derived macrophages (MDMs) [402].

Future studies should determine whether this chronic moderate exercise training can alter the inflammatory responses in in vivo models of sepsis or the susceptibility to viral infections. Overall, this study offers important mechanistic insights into how exercise can cause persistent epigenetic, metabolic, and functional adaptations in macrophages.

4 CHAPTER 4: INVESTIGATING THE SPECIFIC ROLE OF INTERFERON ALPHA (IFN-α) IN INFLAMMATORY RESPONSES TO VIRAL INFECTION AND PAMPS.

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Keywords: IFN I; LCMV; ISGs; Macrophage; Cytokines

4.1 Abstract

Human and mouse genomes comprise over a dozen IFN I subtypes clustered as intronless multigene families. Interestingly, there are 17 in humans and 18 in mice, and they all share the same cell surface receptor and have high sequence homology. This fosters the question of whether they play any qualitatively different roles in antimicrobial responses. Emerging evidence appreciate that they have differential expression patterns, receptor binding affinity, differential sensitivity for feedback inhibitions, etc. However, the functional differences among these subtypes are not fully characterized using in vivo models. In this study, we employed multiple knockout (KO) mice generated through specific deletion of IFN I locus regions to study the differential roles of main IFN Is (IFN-β & IFN–α) in viral infection and PAMP inductions. The KO mice we employed include IFN-β^{only} (IFN-αs and other subtypes are deleted), IFN-βKO (IFN-β is deleted) , TKO (total IFN I locus is deleted) and IFNAR (receptor knockout). We examined the roles of IFN-αs in the viral clearance of LCMV cl-13, a chronic infection model. The ability of viral clearance varied among genotypes. Overall, viral clearance data indicate that IFN-β^{only} is insufficient to control the virus at early stages (d8). This suggests a vital role for IFN-αs in early viral clearance. To understand the underlying mechanisms behind these alterations, we assessed the serum cytokine signatures upon viral infection and in vivo PAMP inductions using flow cytometry-based ELISA and ISG expression levels in BMDMs using RT-qPCR. We also evaluated basal and stress hematopoiesis using spectral flow assays. In vivo cytokine responses in IFN-β^{only} and IFN-βKO upon viral infection was largely intact when compared to WT, while there was a defect in serum cytokines in TKO and IFNARKO (IFN I receptor KO). The loss of IFN-αs and other subtypes altered the serum cytokine and ISG signatures of BMDMs upon PAMP induction when compared to WT. Bone marrow immune cell composition was also altered across genotypes.

Overall, this study showed the importance of IFN-αs in viral clearance of LCMV Cl-13, cytokine and ISG responses, as well as in bone marrow hematopoiesis. Thus, this study contributes significantly to our understanding of the complex IFN I signalling which is one of the main contributors to inflammation. This finding also encouraged the need to study the individual IFN Is in isolation using reliable mice models to uncover their specific roles and targets.

4.2 Introduction

Isaacs and Lindemann discovered interferons in 1957 as an antiviral substance that interferes with the influenza virus infection and named after their effector function interfering with viral replication.[403,404] Later, IFNs were described as small proteins produced by various cell types upon recognizing PAMPs. Nearly two decades later, scientists reported IFNs as a family of cytokines and classified them into three types based on their receptor complexes and signalling pathways: Type I, II and III.[405–407] (Fig. 1). Signalling cascade of all three IFNs and their subtypes results in transcriptional induction or repression of around 2000 Interferons Stimulated Genes (ISGs). The functional outcome of IFN Is is divergent concerning antiproliferative activity, ISG profiles, antiviral, induction kinetics, and immunomodulatory potential. To date, we know that there are 17 type-1 interferons in humans (IFN-B, 13 αs, ε, κ, ω τ, δ and ζ) and 18 in mice (IFN-B, 13 αs ε, κ, ω, τ, δ and ζ) and are clustered in the same chromosome, chromosome 9 in human and 4 in mice.[15] All infected cells can produce IFN Is, but plasmacytoid-dendritic cells (pDCs) are specialized producers of IFN Is.

Although IFN Is share functional similarities, it is unlikely that the evolution would have expanded the gene family unless individual subtypes served unique or distinct purposes during infections. Not surprisingly, a growing body of evidence has now shown that there are functional differences

among IFN I subtypes.[211,408–410] These differences are attributed to their binding affinities, dissociation properties and the binding duration to the receptor. These differences can affect the IFN I induced conformational changes in the receptor, impacting the signalling outcomes or biological functions. IFN-β has the highest binding affinity compared to other subtypes.[411,412]

There are two different LCMV clones: LCMV clone -13 (LCMV Cl-13), which is just three amino acids different from the parent strain LCMV Armstrong (LCMV-Arm) and cause chronic infection and acute infection, respectively. [234,238–241] It has been shown that IFN-α is detected in both LCMV infections; however, Cl-13 infection induced a 3-fold higher IFN-α compared to LCMV-Arm.[242] On the other hand, high levels of IFN-β was detected in CL-13 infection early, while only a minimal level was detected for LCMV-Arm. [413] This suggests that IFN-β may play roles in persisting viral infections and indicates the presence of unique roles for different subtypes.

Interestingly, a study by Van Pesch et al., 2004 characterized all mice IFN-αs invitro and showed that certain IFN I subtypes showed higher biological activities than others (e.g., IFN-α4, IFN-α11, IFN-α12, IFN-β, etc.). This study also showed the correlation between antiproliferative and antiviral properties. These functional differences can arise from their distinct biological activities or expression specificities.[408]

Considering the treatments and therapies, it is known that IFN-β therapies reduce disease severity and progression in autoimmune diseases such as multiple sclerosis.[414] Studies suggest this possibly occurs through the production of anti-inflammatory cytokines[415–418] and reduction in T cell proliferation.[415] Furthermore, in the murine cerebral malaria model, IFN-β treatment reduces disease severity and increased survival by reducing T cell recruitment to the brain.[419] Moreover, it is well-known that IFN-β induces IFNα , which is known for its antiviral activity. Furthermore, IFN-α blockade increased early viral dissemination but did not improve viral control.[420] Besides

viral infection, it is now well appreciated that IFN Is become double-edged swords in cancer.[169] Thus, the evidence appreciates that IFN-β plays both beneficial and detrimental roles depending on the context. This indicates that the roles of these cytokines are complex and IFN-αs and IFN-β are playing distinct roles in terms of antiviral and anti-tumour responses. Further research is warranted to understand how different subtypes work together to produce an effective outcome.

Various mouse models have been used in this field to uncover the complexity of this signalling, including mouse models such as IFN-β-/-, IRF knockout mice (IRF3, 5 or 7), Stat1 and Stat2 knockout mice, IFNAR-/-, etc.[420–428] In addition to knock-out mouse models, distinct approaches such as antibody-mediated specific blockade or mutational analysis have been employed in this area of study.[429–431] However, genetic deletion of the complete IFN-α locus or complete IFN-I (TKO) locus in mice has never been examined.

Based on these previous observations, we designed this study to compare the differences between IFN-β and IFN-α (or others) using mouse models generated via genetic deletion.

Thus, in this study, we employed multiple knockout (KO) mice of IFN I locus to study the roles of IFN-αs in antiviral and PAMP induced inflammatory responses. The KO mice we employed include IFN-β^{only} (IFN-α KO) where the IFN-α locus (this also includes the other IFN I subtypes) is genetically deleted, IFN-βKO (IFN-β is deleted) and TKO (total KO), where the whole IFN-I locus is deleted. (Fig. 5.1) These genetic manipulations were carried out to enable the subsequent re-introduction of individual IFN-α subtypes to make knock-in mice which will serve as reliable models to study the unique roles of IFN-Is independently (Fig. 4.1).

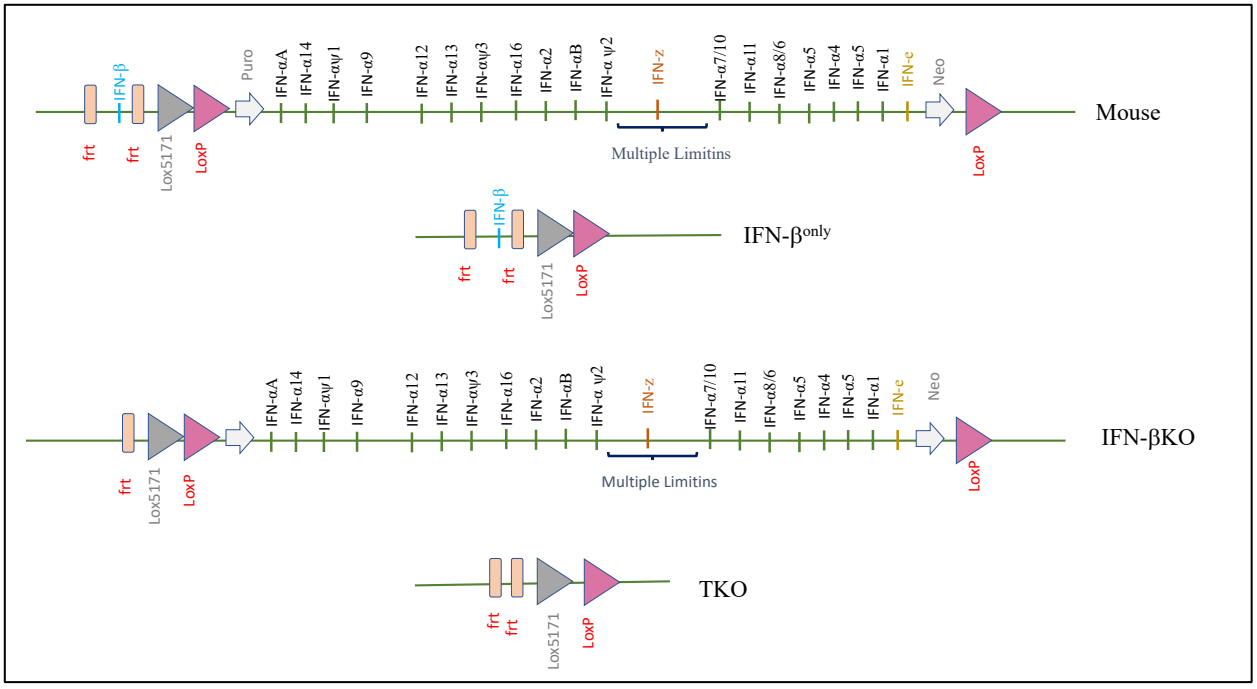


Fig. 4.1: IFN I loci of mouse and the corresponding genetic manipulations of genotypes. IFN I Loci (A), IFN-β^{only} (IFN-α KO) where the total IFN-α locus (this also includes the other IFN Isubtypes) is deleted (B), IFN-βKO (IFN-β is deleted) (C) and TKO (D), where the whole IFN-I locus is deleted. IFN-I genes (green), LoxP (pink triangle), Lox5171 (gray triangle) & Frt (orange quadrangles) elements, grey arrows: Puromycin (Puro) and Neomycin (Neo).

We assessed their ability to clear viral infection using acute (LCMV-Armstrong-data not shown) and chronic (LCMV-Cl-13) LCMV clones. We found that the ability of viral clearance was altered among genotypes. Especially, IFN-β^{only} mice showed reduced viral clearance compared to WT and IFN-βKO and was comparable to TKO and IFNARKO (IFN-I receptor KO).

Furthermore, we also characterized the cytokine signatures upon LCMV viral infection and PAMPs (poly I:C or LPS) inductions in vivo and ISG signatures invitro in BMDMs. In addition, we measured the basal and stress hematopoiesis among these mice. Overall, this study showed the importance of IFN-αs in viral clearance and showed altered cytokine, ISG signatures, and bone marrow hematopoiesis among genotypes. To further understand the specific roles of individual

subtypes in these altered responses, we will generate knock-in mice expressing subtypes in the future.

4.3 Aims and Research questions.

What has been done in the past to uncover the complexity of IFN I signaling?

Several approaches have been used to uncover the complexity of IFN I signalling. This includes mouse models such as IFN-β-/-, IRF knockout mice (IRF3, 5 or 7), Stat1 and Stat2 knockout mice, IFNAR-/-, etc.[420–428] In addition to knock-out mice models, distinct approaches such as antibody-mediated specific blockade, invitro transfection approaches and mutational analysis have been employed in this area of study.[429–431] However, genetic deletion of complete IFN-α locus (IFN-β^{only}mice), individual IFN-αs or complete IFN-I (TKO) locus has never been examined. This is especially important since recent evidence has shown that despite all interferons binding the same receptor (IFNAR), different IFN-Is can activate unique ISGs and exert distinct immune functions.[175,432]

Based on these previous observations, we designed this study to investigate the functional properties of main IFN-αs using multiple mouse models generated via genetic deletion.

Aim I. Research question: Will IFN-β^{only} mice (IFN-αKO) mount an effective response to LCMV Cl-13 infection?

Aim II. Research question: Can IFN-β^{only} (IFN-αKO) BMDMs & mice produce an effective immune response to PAMPs mediated inductions?

Aim III. Research question: Can IFN-β^{only} mice support basal and stress hematopoiesis? IFN Is also regulate the hematopoiesis under basal and stress conditions such as infection or stimulation

with LPS or Poly(I:C).[433–440] Therefore, we hypothesized that IFN-β^{only} mice would have a normal distribution of bone marrow and peripheral leukocytes at rest and during stress-induced hematopoiesis.

4.4 Methods

4.4.1 In vivo injections, serum collection, Bone marrow cells isolation, macrophage differentiation and stimulation

Mice were peritoneally injected with 10 mg / kg body weight LPS or 200 μg poly I:C and incubated for corresponding time points and blood was collected via cardiac puncture. Blood was incubated at RT for 15 minutes and centrifuged at 10000g for 5 minutes. Serum was separated and stored at -80^{o}C. Bone marrow cells from femur was flushed in S+ media (RPMI supplemented with FBS, Glutamate, Pyruvate, Penicillin, streptomycin, non-essential amino acids and β-mercaptoethanol). Red blood cells were lysed. White blood cells were plated on petri-dishes in S+ media containing 25% L929 conditioned media (LCM) for 3 days to allow the monocytes to adhere and differentiate into macrophages. Then the cells were scraped and plated on tissue culture-treated dishes to further differentiate till day 6 in fresh media (S+ and LCM 25%). BMDMS were then stimulated with the corresponding treatments. In this study, we used 100 ng/ml LPS and 25μg poly I:C for invitro stimulations. ***All the viral infections were conducted at UHN, where Elsaesser Heidi (Brooks Lab- our collaborators) performed the viral infection and serum collections.***

4.4.2 Knockout mice generation

Knockout mice were generated by Dr. Ali Abdul-Sater in his postdoc along with Dr. Christian W Schindler in 2012 at Columbia university. (Briefly described in the Apendix A).

4.4.3 RNA extraction and RT-PCR for BMDMs

Total RNA was isolated using BioRad Aurum^{TM} total RNA mini kit and reverse transcribed with M-MuLV reverse transcriptase (New England Bio Labs Inc.) and oligo dT primers (Qiagen) into cDNA. Genes of interest were amplified using the SYBR Green Master mix (Bio-Rad, USA) in the CFX384 Touch Realtime PCR Detection System (Bio-Rad).

4.4.4 Cytokine profiling

Serum samples were diluted five folds in the assay buffer before loading to the plates with beads. According to the manufacturer's instructions, levels of IFN-α, IFN-β , TNF-α, IL-6, IFN-γ, Monocyte chemoattractant protein-1 (MCP-1), Chemokine ligand 5/ CCL5 (RANTES), IL-1β, KC, IL-12p70, IP-10, Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-10 were measured using LEGENDplexTM Mouse anti-virus response Panel. Assay was performed in the Attune NxT flow cytometer (Thermo Fisher). The FCS files generated on the flow cytometer were analyzed using the LEGENDplex™ cloud-based analysis software.

4.4.5 Cytec-Spectral flow cytometry assays on total bone marrow to examine bone marrow basal and stress hematopoiesis

Total bone marrow cells isolated from the LPS (10μg/g) or poly I:C (200μg) injected mice femur were treated for red blood lysis. Then the cells were transferred to 96 well plate, washed in FACS buffer (1X PBS with 2 % FBS). Cell was stained in intracellular fluorescent antibody cocktail made in FACS buffer for 25 min at 4^{o}C. If necessary, cell was fixed in 1X IC fixation buffer at RT for 30 min after staining. Stained cells were then analyzed on flow-cytometry. (See Appendix for detailed protocol)

4.4.6 Statistics

The results are presented as means with standard error of the mean (SEM). Significance is determined by a p-value of less than 0.05. Each experiment was conducted multiple times. N values indicated in the figure captions are biological replicates. When comparing two groups, an unpaired t-test is used. When comparing more than two groups, ANOVAs are conducted and followed by a post-hoc analysis, Tukey test, depending on the type of ANOVA. GraphPad Prism 9.4.1" (San Diego, CA, USA) was used to perform the statistical analysis for all the experiments.

4.4.7 Plaque assay

Vero cells were plated on 6 well plates (400 000 to 500 000 cells per well) and incubated for 24hrs. When the cells were 80-95% confluent, the cell layer was infected with 500 µl of virus diluted in the EMEM media for 1hr, shaking every 15 minutes. After 1hr incubation, virus media was aspirated and a layer of 1: 1 mixture of ME agarose: plaque media (3 ml 1.5 ml of ME agarose: 1 .5 ml of plaque media (2x EMEM (+LGlutamine), FBS, 10ml Pen strep)) was added on top of the cell layer and allowed to set at RT for 10 -15 minutes to set. Then the plates were incubated at 37^{o}C incubator for 6 days and the cell layer was stained with 0.1% crystal violet and plaques were counted, and pfu was calculated.

4.5 Results

Despite the pleiotropism and redundancy of IFN-Is, recent studies have appreciated that each subtype possess distinct characteristics beyond quantitative differences.[294, 268,295,296, 297,298,299, 441,442, 278–281] In this study, we examined the effects of IFN-α vs. IFN-β deletion on viral clearance and PAMPs (pathogen-associated molecular pattern) mediated responses. Viral clearance and cytokine signatures upon LCMV (Armstrong-*data not shown* and Cl-13) infection were assessed at multiple days after infections. In vivo PAMP inductions involved peritoneal injection of poly I:C (200 μg) or LPS (10 mg/ kg of mouse body weight) Serum cytokine signatures were characterized using flow-cytometry based Elisa. Bone marrow derived macrophages (BMDMs) were stimulated using poly I:C (25 μg/ml) or LPS (100 ng/ml) and ISG signatures were characterized at the mRNA level using RT-PCR. Bone marrow immune cellular composition was characterized using multi-colour spectral Flow-cytometry.

For Aim I, only the LCMV cl-13 viral load data and serum cytokine signatures are presented in this thesis, as I did not perform the other viral experiments directly. ***All the viral infections were conducted at UHN, where Elsaesser Heidi (Brooks Lab- our collaborators) performed the viral infection and serum collections.***

4.5.1 Aim I. Will FN-β^{only} mice mount an effective response to LCMV Cl-13 infection?

The role of IFNs in viral control is well known. Recent research has suggested IFN I subtypes (e.g. IFN-α and IFN-β) may respond to viral infections differently. IFN-α was shown to support restricting early viral dissemination, while IFN-β is implicated in prolonging infections.[242,420]

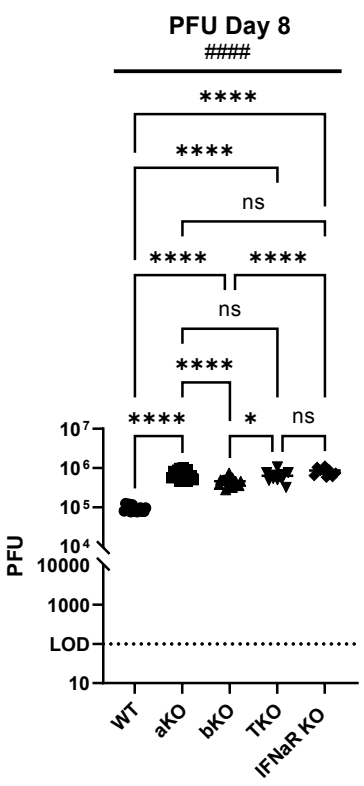
We measured the serum viral loads upon LCMV cl-13 infection at three different time points (Days 8, 30 and 60). LCMV Cl-13 infection is persistent, lasts over 60 days, and peaks around 8-10 days.[443] Based on these previous observations, we chose time points for this study. We employed

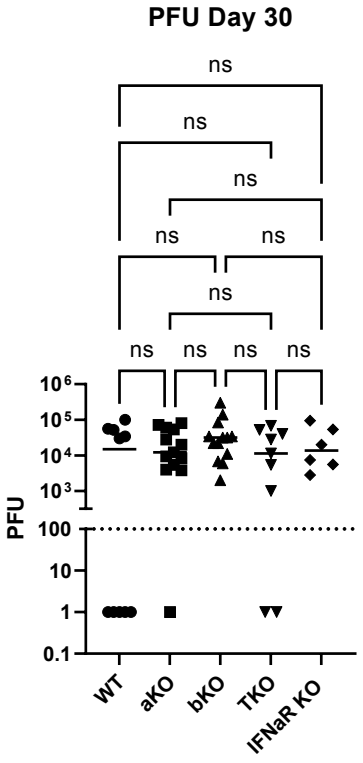
WT (C57BL/6), IFN-β^{only}, IFN-βKO (lacks IFN-β) and TKO mice (which lacks the whole Type 1 IFNs locus). All mice strains were bled at indicated time points following infection, and serum was extracted.

All KO genotypes had significantly higher viral load compared to WT at Day 8 **(Fig. 4.2A).** Interestingly, IFN-β^{only} viral load was comparable to TKO and IFNARKO. This could indicate the importance of IFN-α in viral clearance at the early stages (day 8 **(fig. 4.2A)).**[242,420] TKO and IFNARKO also show comparable viral load, which is unsurprising; however, comparable viral load in IFN-β^{only} to TKO and IFNAR is an interesting observation.

In addition, in the Day 8 serum samples, viral load in IFN-βKO is also high compared to WT but significantly lower compared to IFN-β^{only}. Moreover, at day 30 **(Fig.4.2B)**, no significant differences in pfu were observed among genotypes, but the chronic infection was evident as the viral load was comparable. However, at day 60 **(Fig. 4.2C),** most pfu numbers went down lower or close to the threshold; therefore, no significance was detected. Fig. **4.2 D and E** show the time course of viral clearance.

Overall, given that IFN-β has been implicated in chronic viral persistence,[242,244] this is interesting as this may indicate that IFN-β^{only} IFN-α (and others) only are not sufficient to control the virus at early stages (d8**(Fig. 4.2A)**), but then at late stages (d60 **(Fig. 4.2A)**), they appear to be enough to yield viral clearance albeit not in all mice, as opposed to TKO and IFNARKO. Curiously a few TKO cleared the virus, which may indicate a yet-to-be-identified role for IFNAR in controlling viral infections.


PFU Day 8
####
****
****
ns
****
****
ns
****
****
*
ns
PFU
10^7
10^6
10^5
10^4
10000
1000
LOD
10
WT
aKO
bKO
TKO
IFNaR KO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ns
ns
ns
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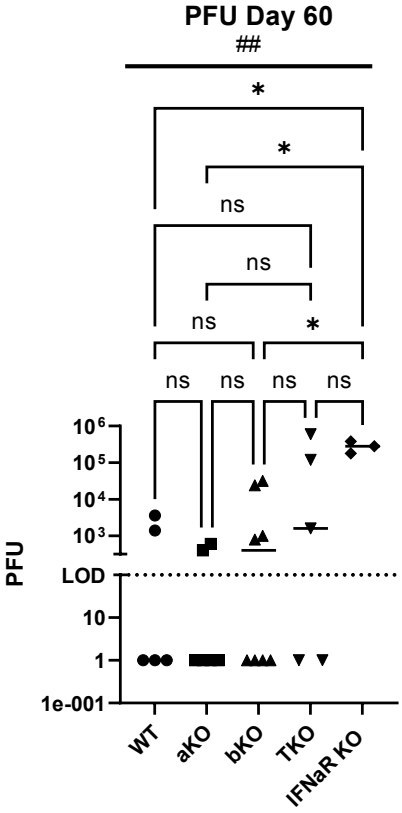
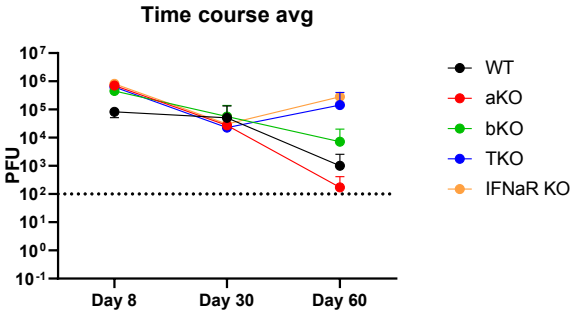
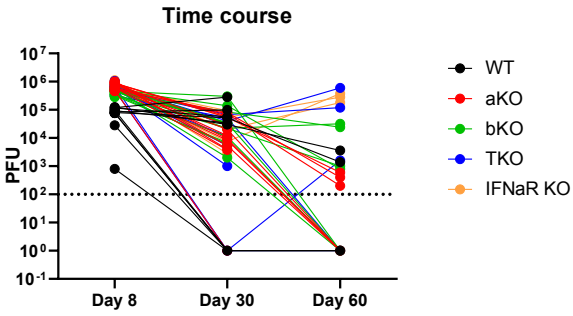
ns
ns
ns
ns
ns
PFU
10^6
10^5
10^4
10^3
100
10
1
0.1
WT
aKO
bKO
TKO
IFNaR KO


PFU Day 60
##
*
*
ns
ns
ns
*
ns
ns
ns
ns
PFU
10^6
10^5
10^4
10^3
LOD
10
1
1e-001
WT
aKO
bKO
TKO
IFNaR KO


Time course avg
PFU
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
10^{-1}
Day 8
Day 30
Day 60
WT
aKO
bKO
TKO
IFNaR KO


Time course
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10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
10^{-1}
Day 8
Day 30
Day 60
WT
aKO
bKO
TKO
IFNaR KO

Fig. 4.2: Viral loads of WT, IFN-β^{only}, IFN-βKO, TKO and IFNARKO mice upon chronic LCMV infection (LCMV Cl-13). Mice were infected with LCMV Cl-13, which provides the chronic infection model. Viral load in the serum was measured using plaque assays on Vero cells. Plaque forming unites (pfu) that represent viral load on A) Day 8, B) Day 30 and C) Day 60 are shown. D) the time course of individual mouse viral load and the E) the average time course of vital load. Stats: one-way Anova (n= 5-15), alpha: 0.05. * Indicating a significant difference from multiple comparisons. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. # indicates the P value summary-anova.

These observations indicate the value of examining the underlying mechanisms and the unique roles of IFN-I subtypes in antiviral response. To study the mechanisms behind the altered antiviral properties of these genotypes, we further examined how these genetic alterations affect the cytokine signatures in mice blood upon LCMV-Cl-13 infection.

4.5.2 Cytokine signatures upon LCMV-Cl-13 infection

This experiment aimed to examine if IFN-β^{only} and IFN-αs (and others) only are enough to promote systemic cytokine production upon LCMV-Cl-13 infection. Serum samples were collected after 8, 30 and 60 days and serum cytokine levels were assessed.

We used Legendplex^{TM} Mouse Anti-Virus Response Panel for this analysis. It is a flow cytometry-based multiplex Elisa uses fluorescence–encoded beads. It can measure thirteen types of cytokines, including type 1 and type II interferons (IFN-α, β, γ), interleukins (1β, 6, 10, 12), CXCL1 (KC), CXCL10 (IP10), CCL2 (MCP-1), CCL5 (RANTS), TNF-α and GM-CSF **(Fig. 4.3).** [444,445]

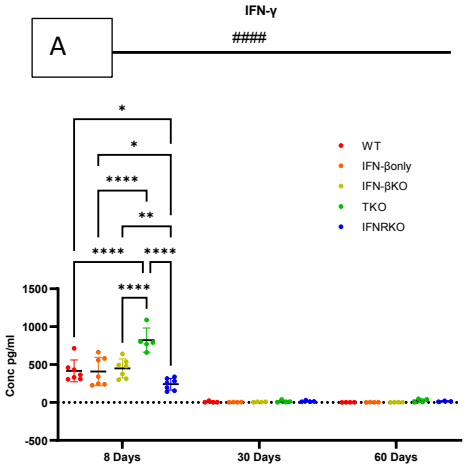
First, considering the differences among genotypes, overall, significant differences were only observed for IFN-γ, IFN-α, TNF-α, MCP-1, IL-1β and IP-10. **(Fig. 4.3 A, B, E, F, I, J).** Independent comparisons showed no significant differences in cytokine levels between WT and IFN-β^{only} or IFN-β^{only} and IFN-βKO or WT and IFN-βKO.

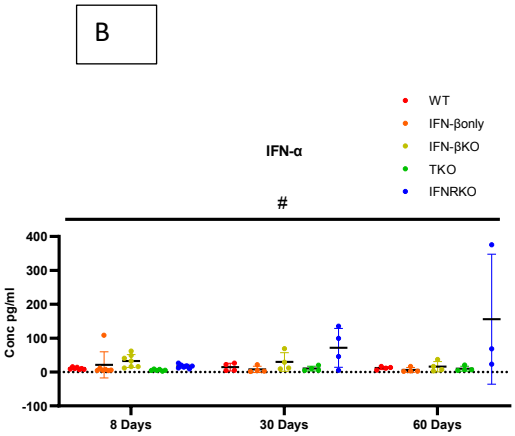
Surprisingly, IFN-γ level in TKO was significantly increased compared to all genotypes. This is interesting because IFN-γ is a type II IFN, follows different but partially overlapping signalling

pathways. Furthermore, IFN-γ in IFNARKO was significantly reduced compared to all other genotypes, including TKO **Fig. 4.3 A**. This observation could suggest that there are differences between TKO and IFNAR. Additionally, GMCSF in IFNAR was significantly high and RANTES was lower in TKO and IFNAR than IFN-βKO **Fig. 4.3 K**. RANTS was also lower in IFNAR compared to WT.

Overall, cytokine responses in IFN-β^{only} and IFN-βKO were largely intact when compared to WT, while there was a defect in TKO and IFNARKO. This may suggest that IFN-β^{only} and IFN-α^{only} are enough to promote systemic cytokine production. However, interestingly, CD8 T cell cytokine production was significantly impaired in IFN-β^{only} compared to WT.(*data not shown*)

In addition, most of the cytokines showed a decreasing trend with time from day 8, which shows their reduction in expression as the virus clears, except GMCSF, RANTES, IL-12 and IL-10 (**Fig. 4.3 G, H, K)** suggesting that their expression may be prolonged during the antiviral response. Furthermore, considering the low levels of most of these cytokines detected from LCMV-infected mice serum and the signalling pathways' complexity and redundancy, we chose to use isolated induction models with poly I: C or LPS in the following sections to assess cytokine signatures.


A
IFN-γ
####
*
*
****
**
****
****
****
WT
IFN-βonly
IFN-βKO
TKO
IFNRKO
Conc pg/ml
1500
1000
500
0
-500
8 Days
30 Days
60 Days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IFN-α
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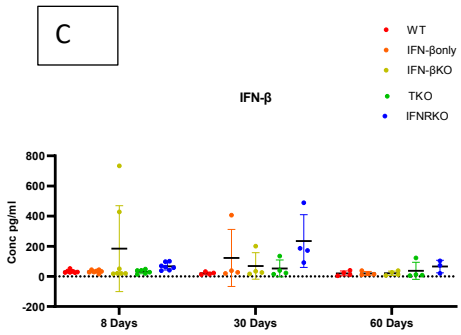
TKO
IFNRKO
Conc pg/ml
400
300
200
100
0
-100
8 Days
30 Days
60 Days


C
IFN-β
WT
IFN-βonly
IFN-βKO
TKO
IFNRKO
Conc pg/ml
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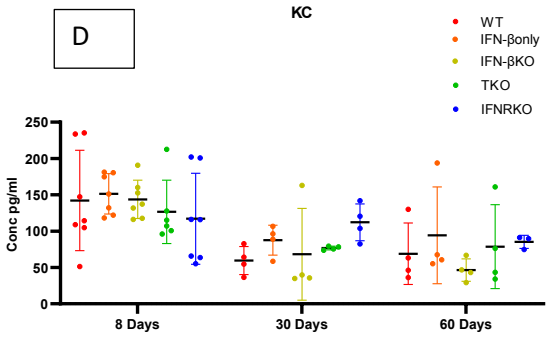
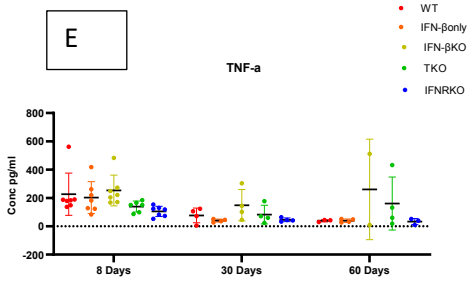
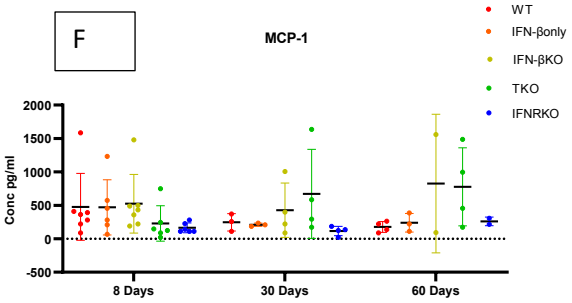
600
400
200
0
-200
8 Days
30 Days
60 Days


D
KC
WT
IFN-βonly
IFN-βKO
TKO
IFNRKO
Conc pg/ml
250
200
150
100
50
0
8 Days
30 Days
60 Days


E
TNF-a
WT
IFN-βonly
IFN-βKO
TKO
IFNRKO
Conc pg/ml
800
600
400
200
0
-200
8 Days
30 Days
60 Days


F
MCP-1
WT
IFN-βonly
IFN-βKO
TKO
IFNRKO
Conc pg/ml
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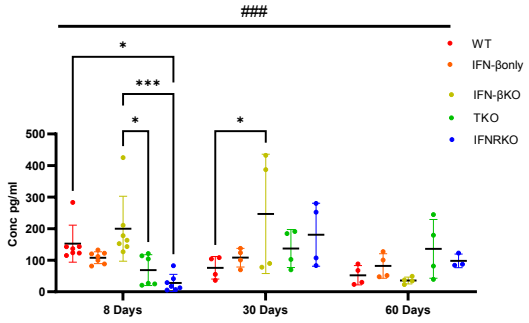
1500
1000
500
0
-500
8 Days
30 Days
60 Days

G


RANTES
###
WT
IFN-βonly
IFN-βKO
TKO
IFNRKO
Conc pg/ml
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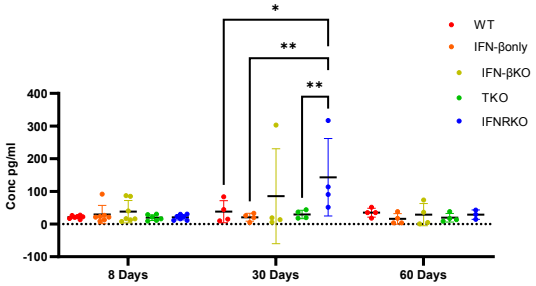
30 Days
60 Days

H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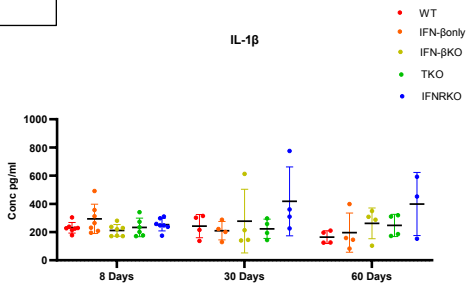
WT
IFN-βonly
IFN-βKO
TKO
IFNRKO
Conc pg/ml
8 Days
30 Days
60 Days

I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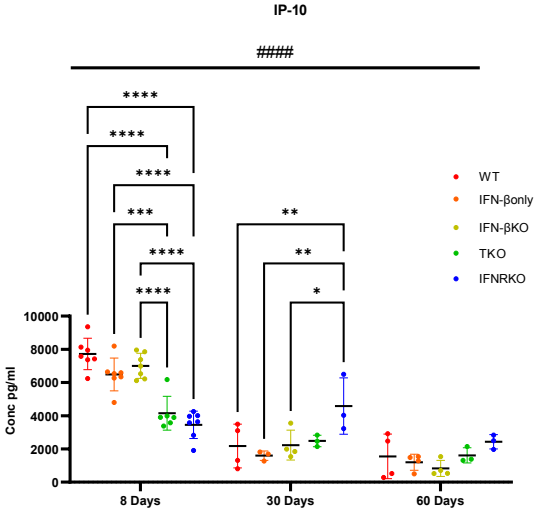
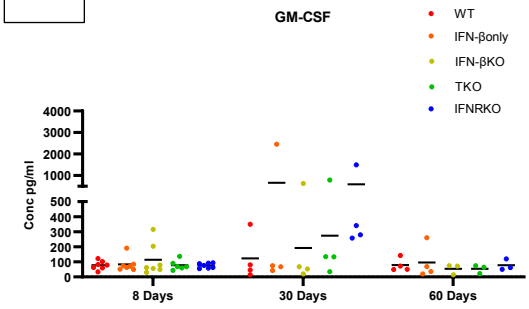
WT
IFN-βonly
IFN-βKO
TKO
IFNRKO
Conc pg/ml
8 Days
30 Days
60 Days

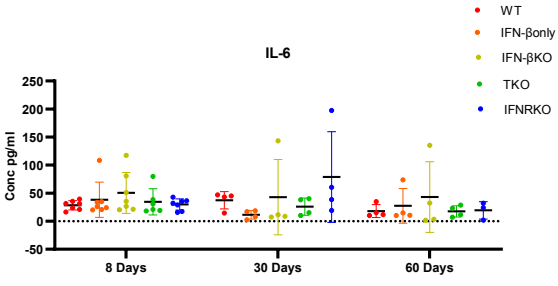
J


IP-10
####
WT
IFN-βonly
IFN-βKO
TKO
IFNRKO
Conc pg/ml
8 Days
30 Days
60 Days

K


GM-CSF
WT
IFN-βonly
IFN-βKO
TKO
IFNRKO
Conc pg/ml
8 Days
30 Days
60 Days

L


IL-6
WT
IFN-βonly
IFN-βKO
TKO
IFNRKO
Conc pg/ml
8 Days
30 Days
60 Days

M

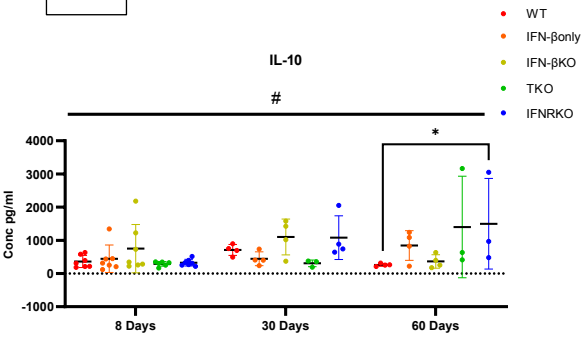

IL-10
#
WT
IFN-βonly
IFN-βKO
TKO
IFNRKO
Conc pg/ml
8 Days
30 Days
60 Days

Fig. 4.3: Cytokine levels in the serum upon LCMV-Cl-13 infection. C57 BL/6 (WT), IFN-β^{only} (IFN-α KO), IFN-βKO, TKO, IFNARKO mice were infected LCMV Cl-13 and incubated for indicated times. Serum levels of cytokines were examined via flow cytometry-based Elisa experiment (legend plex assays). Serum levels of A) IFN-γ, B) IFN-α, C) IFN-β, D) KC, E) TNF-α, F) MCP-I, G) RANTES, H) IL-12, I) IL-1β, J) IP-10, K) GM-CSF, L) IL-6, M) IL-10 are shown. Each symbol represents individual mouse. Each symbol represents an individual mouse (n=3-6 per group). Data were compared by multiple comparisons from a 2-way anova. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. # indicates the P value summary-interaction.

Aim II. Can IFN-βonly (IFN-αKO) BMDMs & mice produce an effective immune response to PAMPs mediated induction?

4.5.2.1 Cytokine levels in the serum upon poly I:C IP injection: genotypes- WT, IFN-β^{only}, IFN-βKO and TKO

Poly I:C is a synthetic double-stranded RNA used in research to induce TLR3 signalling and IFN I responses. TLR3 signalling induces type 1 IFNs both in vitro and in vivo. TLR3 is expressed broadly in myeloid and non-myeloid cells, including macrophages. It induces IFN I expression through TRIF-mediated Signalling. Poly I:C induces IFN I expression in two to four hours.(18–20) IFN-β increase upon poly I:C injection precedes ISG increase in plasma.[446,448–451] Quantifying the IFN I subtypes expression has been done in several studies.[203,452] Similarly, quantifying ISGs or cytokine expressions upon specific IFN I induction will also be key steps in uncovering the unique signalling properties of the IFN-I subtypes.

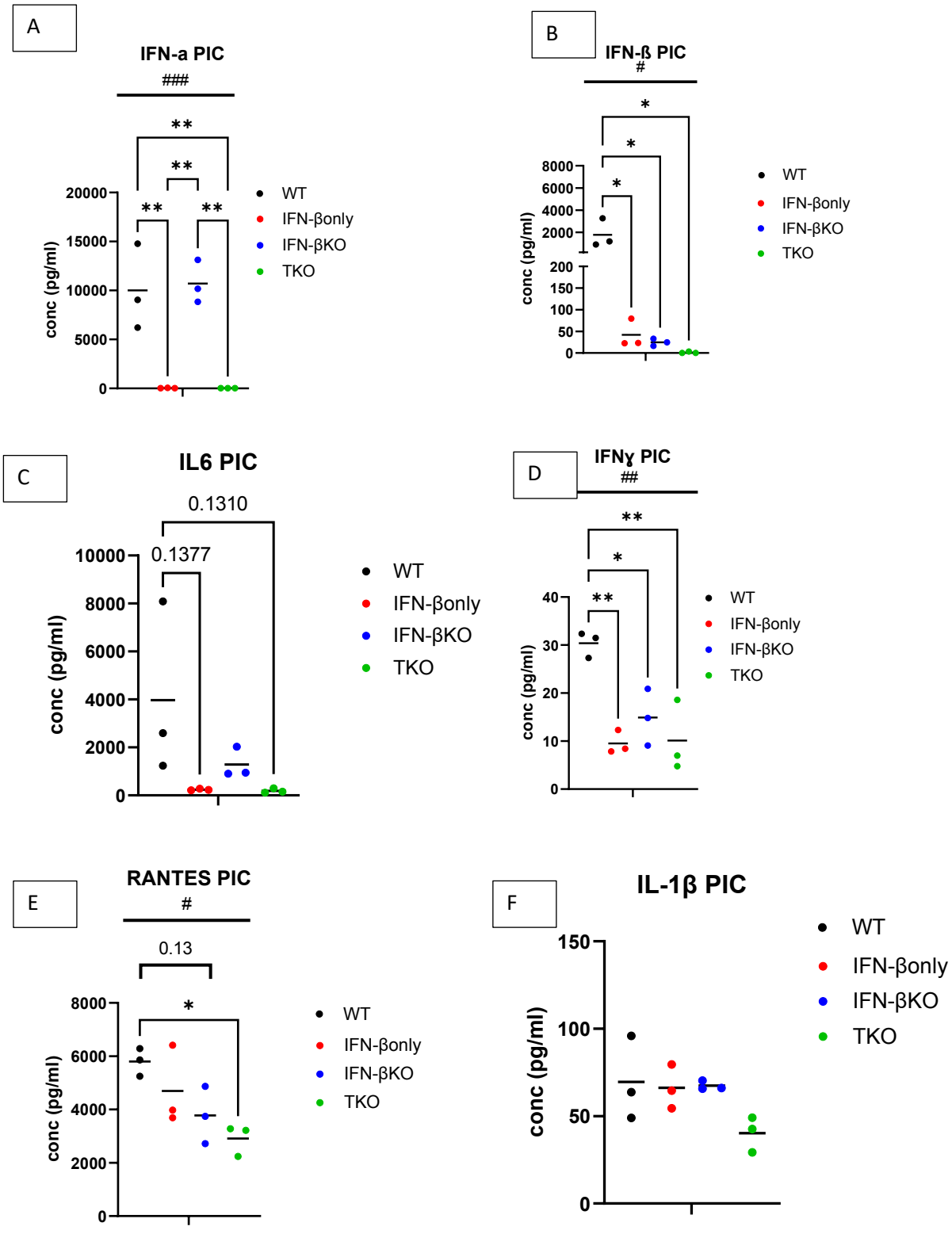
Upon poly I:C injection, serum cytokine levels were measured using Legendplex^{TM} Mouse Anti-Virus Response Panel. It is a flow cytometry-based multiplex Elisa uses fluorescence–encoded beads. It can measure thirteen types of cytokines, including type 1 and type II interferons (IFN-α, β, γ), interleukins (1β, 6, 10, 12), CXCL1 (KC), CXCL10 (IP10), CCL2 (MCP-1), CCL5 (RANTS), TNF-α and GM-CSF **(Fig. 4.4).** [444,445] This experiment compared the antiviral cytokine

responses among WT, IFN-β^{only}, IFN-βKO, and TKO following poly I:C injections. The serum was collected at a 3hr and the serum cytokine levels were analyzed.

We measured serum cytokine levels to examine this. Here we made the comparison in two different ways. First WT vs. IFN-β^{only}. In this comparison, no significant IFN-α expression was observed in IFN-β^{only}. Serum IFN-β was significantly lower in IFN-β^{only} compared to WT. Furthermore, significant decrease in IFN-γ MCP-1 and KC **Fig. 4.4 D, K, L,** was observed but not for IL-6, RANTS, IL-12, IP-10, IL-10, TNF-α and GM-CSF (**Fig. 4.4 C, E, G, I, J, M)**. IL-1β (**Fig. 4.4 F)** did not show any significant changes. Furthermore, a significantly high level of IFN-α (**Fig. 4.4 A)** was observed in IFN-βKO, which was comparable to WT levels, but IFN-β level (**Fig. 4.4 B)** in IFN-β^{only} was not noticeably higher. Given that IFN-β is expressed in the early phase of the response, this increased IFN-αs in IFN-βKO may be trying to compensate for the absence of IFN-β. Furthermore, Only KC showed strikingly high levels in IFN-β^{only} than WT or IFN-βKO (**Fig. 4.4 L**). IL-6, IFN- γ, RANTS, IL-1β IL-12, IP-10, IL-10, TNF-α, MCP-1, and GM-CSF were not significantly different among genotypes (**Fig. 4.4 C- K, M**).

Considering WT vs. IFN-βKO, no significant differences were observed for IFN-α levels **(Fig. 4.4A)**. IL-6, RANTS, IL-1β, IL-12, IP-10, IL-10, TNF-α, KC, and GM-CSF were not significantly different (**Fig. 4.4 C, E, G-J, L-M)**. IFN-γ and MCP-1 were significantly low in IFN-βKO compared to WT **(Fig. 4.4 D, K)**.

Focusing on TKO, similar to what we observed in gene expression, IFN-α and IFN-β **(Fig. 4.4 A-B)**were not detectable in TKO. Several ISGs showed up at lower levels in the serum as well; this could again indicate the influence of other signaling pathways in their expression.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IFN-a PIC
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**
**
**
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20000
15000
10000
5000
0
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WT
IFN-βonly
IFN-βKO
TKO
B
IFN-ß PIC
#
*
*
*
8000
6000
4000
2000
200
150
100
50
0
conc (pg/ml)
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IFN-βonly
IFN-βKO
TKO
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0.1377
10000
8000
6000
4000
2000
0
conc (pg/ml)
WT
IFN-βonly
IFN-βKO
TKO
D
IFNɣ PIC
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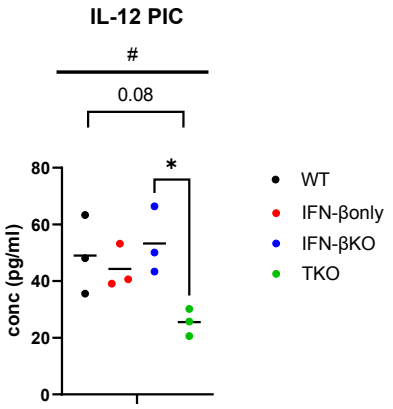
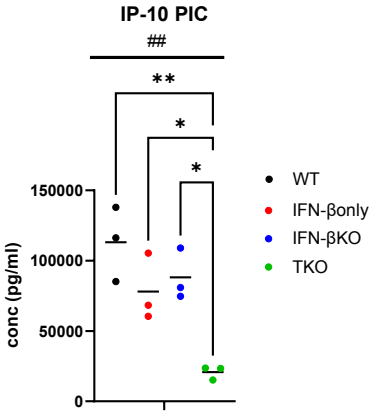
40
30
20
10
0
conc (pg/ml)
WT
IFN-βonly
IFN-βKO
TKO
E
RANTES PIC
#
0.13
*
8000
6000
4000
2000
0
conc (pg/ml)
WT
IFN-βonly
IFN-βKO
TKO
F
IL-1β PIC
150
100
50
0
conc (pg/ml)
WT
IFN-βonly
IFN-βKO
TKO

G


IL-12 PIC
#
0.08
*
conc (pg/ml)
80
60
40
20
0
WT
IFN-βonly
IFN-βKO
TKO

H


IP-10 PIC
##
**
*
*
conc (pg/ml)
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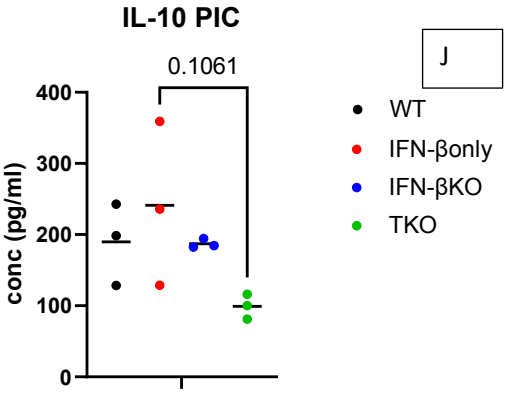
100000
50000
0
WT
IFN-βonly
IFN-βKO
TKO

I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conc (pg/ml)
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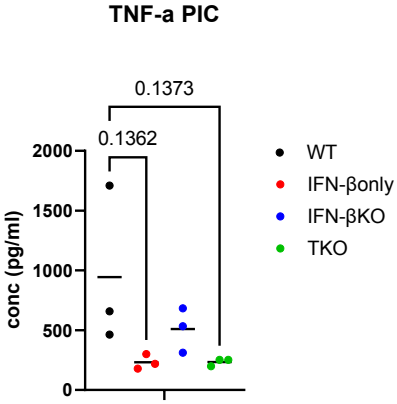
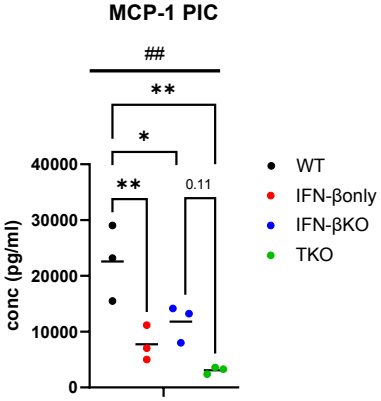
300
200
100
0
WT
IFN-βonly
IFN-βKO
TKO

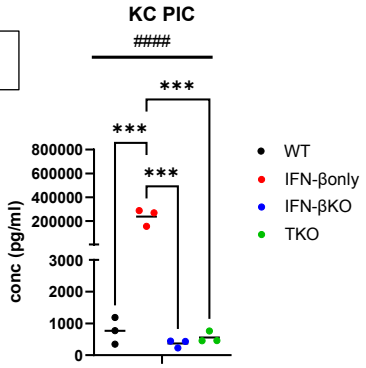
J


TNF-a PIC
0.1373
0.1362
conc (pg/ml)
2000
1500
1000
500
0
WT
IFN-βonly
IFN-βKO
TKO

K


MCP-1 PIC
##
**
*
**
0.11
conc (pg/ml)
40000
30000
20000
10000
0
WT
IFN-βonly
IFN-βKO
TKO

L


KC PIC
####
***
***
***
conc (pg/ml)
800000
600000
400000
200000
3000
2000
1000
0
WT
IFN-βonly
IFN-βKO
TKO

M

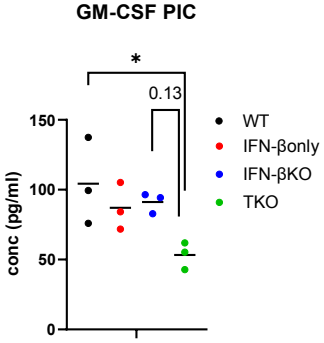

GM-CSF PIC
*
0.13
conc (pg/ml)
150
100
50
0
WT
IFN-βonly
IFN-βKO
TKO

Fig. 4.4: Cytokine levels in serum upon PIC IP injection. C57 BL/6 (WT), IFN-β^{only} (IFN-α KO), IFN-βKO and TKO mice were intraperitoneally injected with (200 ug) PIC and incubated the mice for indicated times. Serum levels of cytokines were examined via flow cytometry-based Elisa experiment (legend plex assays). A) Serum levels of IFN-α B) IFN-β C) IL-6, D) IFN-γ, E) RANTES F) IL-1β G) IL-12. H) IP-10, I) IL-10, J) TNF-α, K) MCP-1, L) KC, M) GMCSF are shown. Each symbol represents an individual mouse (n=3 per group). Data were compared by multiple comparisons from a one-way anova. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$# indicates the P value summary-anova.

4.5.2.2 Serum cytokine levels upon LPS IP injection: genotypes- WT, IFN-β^{only}, IFN-βKO.

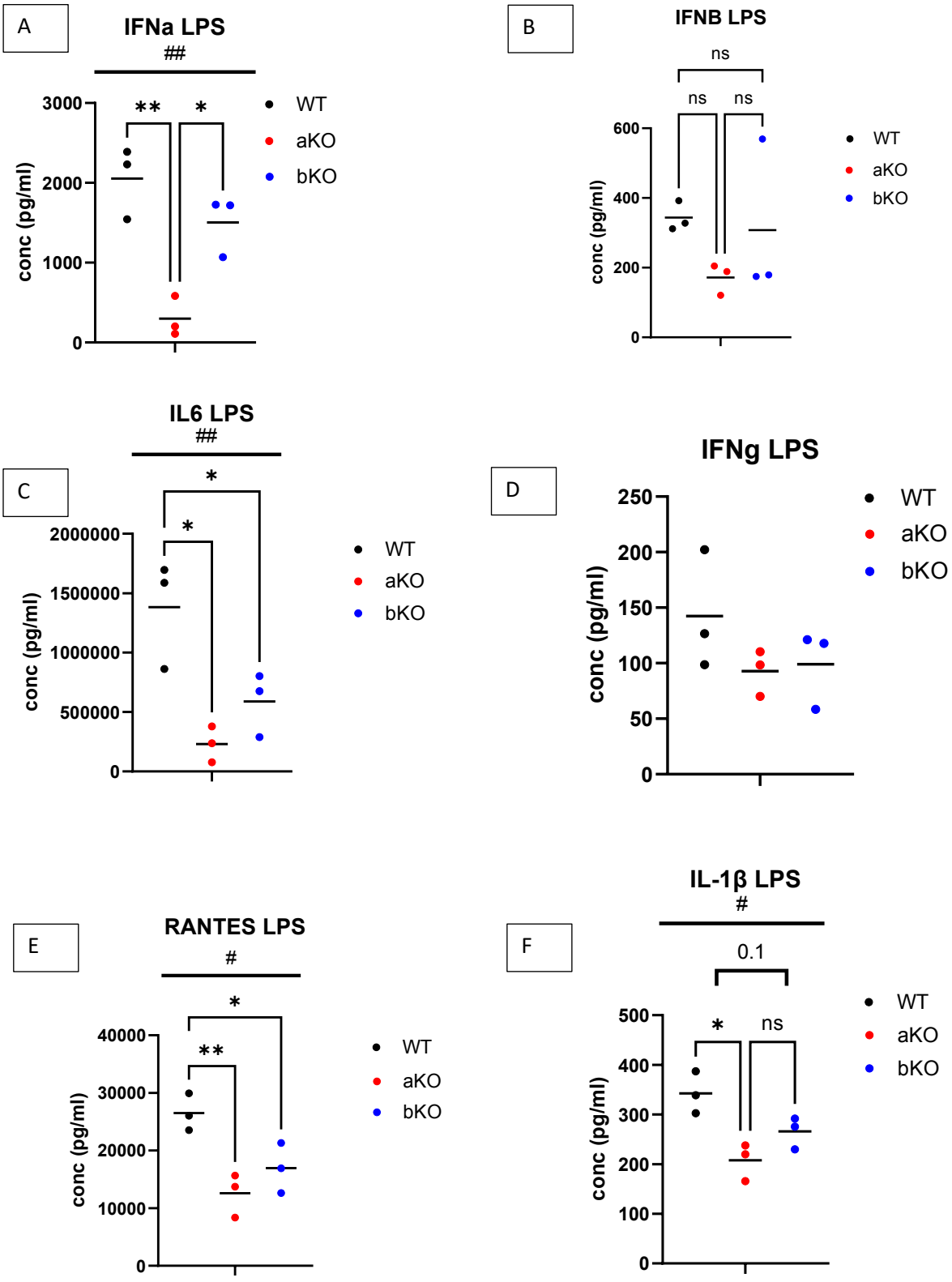
LPS (lipopolysaccharide), is a cell wall component of gram-negative bacteria. It can activate the innate immune response by binding to TLR4, a toll-like receptor, on the surface of host cells. This binding leads to the activation of the downstream signalling molecule TRIF, which then triggers the interferon response.[453]

We measured the cytokine levels in the serum upon LPS injection using the legend plex mouse antiviral assay panel. Again, we made a comparison in two different ways. First WT vs. IFN-β^{only}. In this comparison, no significant IFN-α expression was observed in IFN-β^{only} as for the poly I:C injection **(Fig. 4.5 A)**.. Unlike for poly I:C injection, no significant change in FN-β was detected for IFN-β^{only} when compared to WT **(Fig. 4.5 B)**. Furthermore, TNF-α, IP-10, IL-12 and IL-10 were significantly increased in IFN-β^{only} compared to WT and MCP-1 was increased albeit in a non-significant manner ($p=0.07$) **(Fig. 4.5 G-J)**. No significant difference was observed for IL-6, IFN-γ, RANTS, KC and GM-CSF, (**Fig. 4.5 C-E, L-M)**. Like in the poly I:C injection, IL-1β was not different.

Considering WT vs. IFN-βKO: neither IFN-α nor IFN-β were significantly different (**Fig. 4.5 A-B)**. IL-6, RANTES and IP-10 (**Fig. 4.5 C, E, H)** were significantly decreased compared to WT, but no differences were found between IFN-β^{only} and IFN-βKO. IFN-γ, IL-1β, IL-10, TNF-α, MCP-1, KC and GMCSF were not different among genotypes ((**Fig. 4.5 C, F, I-K, L-M)**).

Comparing IFN-β^{only} vs. IFN-βKO: similar to poly I:C injection, a significantly high level of IFN-α was observed in IFN-βKO which was comparable to WT levels, but IFN-β levels in IFN-β^{only} was not noticeably higher (**Fig. 4.5 A, B)**. This was similar to PIC injection, and this could again suggest that this increased IFN-αs in IFN-βKO may be trying to complement the absence of IFN-β. IL-12 and TNF-α were higher in IFN-βKO than IFN-β^{only} (**Fig. 4.5 G, J)**. However, IL-6, FN-γ, RANTES, IL-1β, IP-10, IL-10, MCP-1, KC and GMCSF were not different (**Fig. 4.5 C-F, H, K, L)**.

Overall, the differential expression of cytokines in the serum from the poly I:C and LPS in vivo induction indicates qualitative differences between IFN-β^{only} and IFN-βKO. Furthermore, IFN Is also have important functions in hematopoiesis.


A
IFNa LPS
##
**
*
conc (pg/ml)
3000
2000
1000
0
WT
aKO
bKO
B
IFNB LPS
ns
ns
ns
conc (pg/ml)
600
400
200
0
WT
aKO
bKO
C
IL6 LPS
##
*
*
conc (pg/ml)
2000000
1500000
1000000
500000
0
WT
aKO
bKO
D
IFNg LPS
conc (pg/ml)
250
200
150
100
50
0
WT
aKO
bKO
E
RANTES LPS
#
*
**
conc (pg/ml)
40000
30000
20000
10000
0
WT
aKO
bKO
F
IL-1β LPS
#
0.1
*
ns
conc (pg/ml)
500
400
300
200
100
0
WT
aKO
bKO

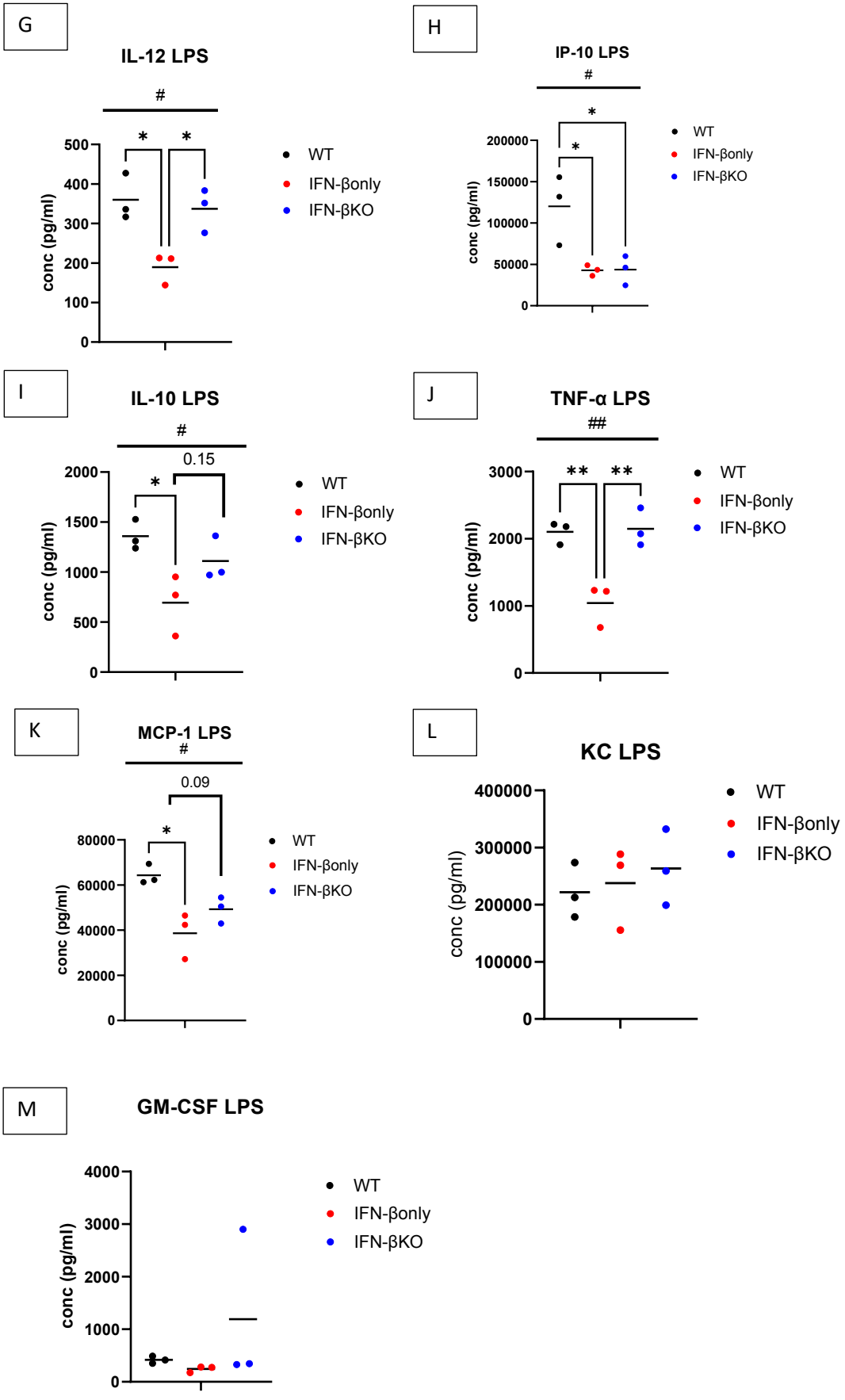

G
IL-12 LPS
#
*
*
conc (pg/ml)
500
400
300
200
100
0
WT
IFN-βonly
IFN-βKO
H
IP-10 LPS
#
*
*
conc (pg/ml)
200000
150000
100000
50000
0
WT
IFN-βonly
IFN-βKO
I
IL-10 LPS
#
0.15
*
conc (pg/ml)
2000
1500
1000
500
0
WT
IFN-βonly
IFN-βKO
J
TNF-α LPS
##
**
**
conc (pg/ml)
3000
2000
1000
0
WT
IFN-βonly
IFN-βKO
K
MCP-1 LPS
#
0.09
*
conc (pg/ml)
80000
60000
40000
20000
0
WT
IFN-βonly
IFN-βKO
L
KC LPS
conc (pg/ml)
400000
300000
200000
100000
0
WT
IFN-βonly
IFN-βKO
M
GM-CSF LPS
conc (pg/ml)
4000
3000
2000
1000
0
WT
IFN-βonly
IFN-βKO

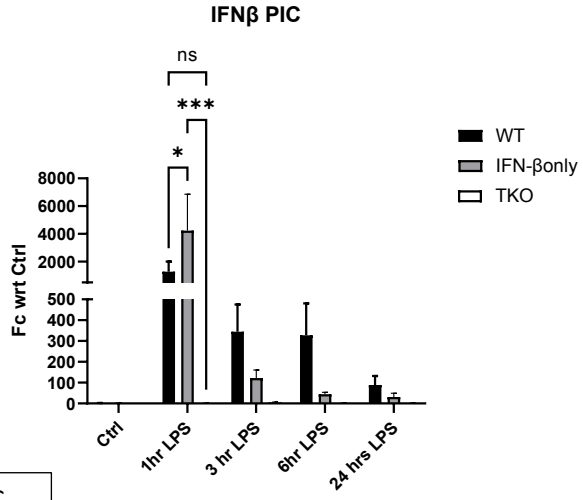
Fig. 4.5: Cytokine levels in serum upon PIC LPS injection. C57 BL/6 (WT), IFN-β^{only} (IFN-α KO) and IFN-βKO mice were intraperitoneally injected with (200 ug) PIC and incubated the mice for indicated times. Serum cytokines levels were examined via flow cytometry-based Elisa experiment (legend plex assays). A) Serum levels of IFN-α B) of IFN-β C) IL-6, D) IFN-γ, E) RANTES F) IL-1β G) IL-12. H) IP-10, I) IL-10, J) TNF-α, K) MCP-1, L) KC, M) GMCSF are shown. Each symbol represents an individual mouse (n=3 per group). Data were compared by multiple comparisons from a one-way anova. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. # indicates the P value summary-anova.

4.5.2.3 Gene expression levels of ISGs in BMDMs upon Poly I:C treatment.

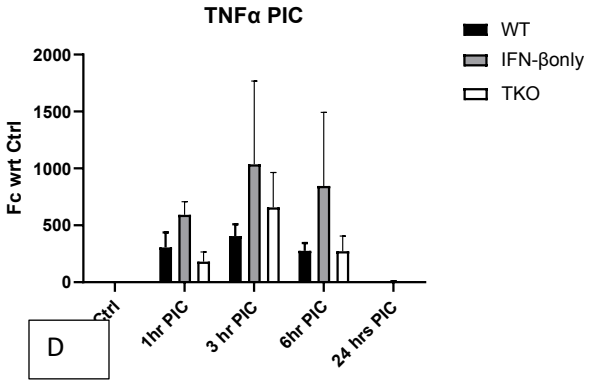
In this study, we stimulated the BMDMs of IFN-β^{only}, WT and TKO with poly I:C and characterized the ISG signatures using RT-PCR. The ISGs measured include MX Dynamin Like GTPase 1 (MX1), interferon stimulated gene 15 (ISG-15), Bone Marrow Stromal Cell Antigen 2 (BST2), RIG-I (DX58), protein kinase R (PKR), CXCL10, IRF1, IRF7, IFN-β, and the cytokine TNF-α **(Fig. 4.6).** Expression levels of ISGs differed among genotypes (IFN-β^{only}, WT, and TKO). For example, we found that the genes IFN-β, PKR, IRF7, MX1A, and DX58 were significantly more highly expressed in the IFN-β^{only} when compared to the WT **(Fig. 4.6 A, E, H- J).** In contrast, the gene IRF-1 was significantly downregulated in the IFN-β^{only} than WT (Fig. 4.7).

Focusing on TKO, as expected, IFN-β or IFN-α were not detectable in TKO **(Fig. 4.6 A,** B). However, several interferon-stimulated genes (ISGs) were expressed at lower levels. These ISGs can also be regulated by other signalling pathways, which likely contributed to the their lower levels in the TKO, which is not unexpected.[232] (Fig. 1) RT-PCR could not accurately determine the IFN-α expression levels because the expression levels were low and the cycle numbers were too high, similar to the no reverse transcription controls (NRTs). Fig. S2 represents the same data in a different format to better evaluate the kinetics of these ISGs upon poly I:C induction at RNA level.

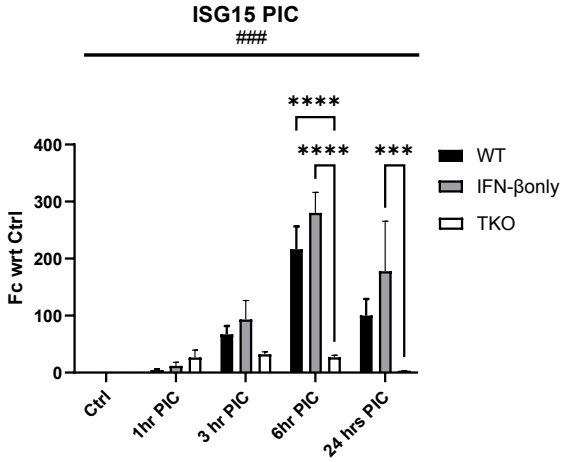
A


IFNβ PIC
ns
***
*
Fc wrt Ctrl
8000
6000
4000
2000
500
400
300
200
100
0
Ctrl
1hr LPS
3 hr LPS
6hr LPS
24 hrs LPS
WT
IFN-βonly
TKO

B


TNFα PIC
Fc wrt Ctrl
2000
1500
1000
500
0
Ctrl
1hr PIC
3 hr PIC
6hr PIC
24 hrs PIC
WT
IFN-βonly
TKO

C


ISG15 PIC
###
****
****
***
Fc wrt Ctrl
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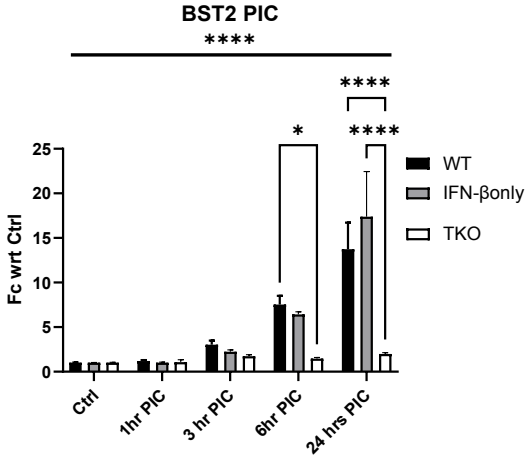
300
200
100
0
Ctrl
1hr PIC
3 hr PIC
6hr PIC
24 hrs PIC
WT
IFN-βonly
TKO

D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****
****
*
****
Fc wrt Ctrl
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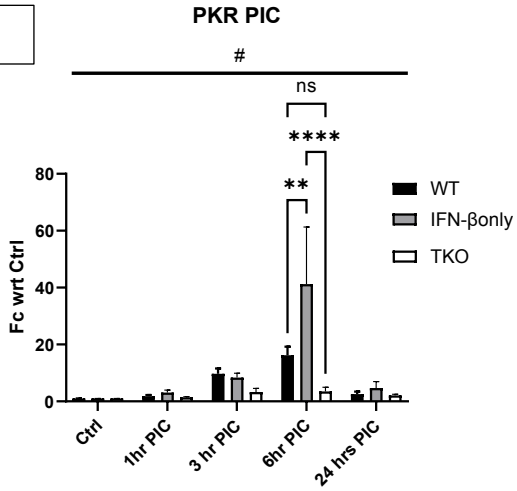
20
15
10
5
0
Ctrl
1hr PIC
3 hr PIC
6hr PIC
24 hrs PIC
WT
IFN-βonly
TKO

E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#
ns
****
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Ctrl
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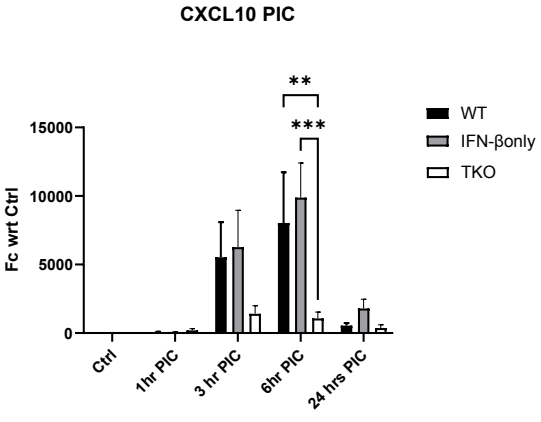
6hr PIC
24 hrs PIC
WT
IFN-βonly
TKO

F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6hr PIC
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WT
IFN-βonly
TKO

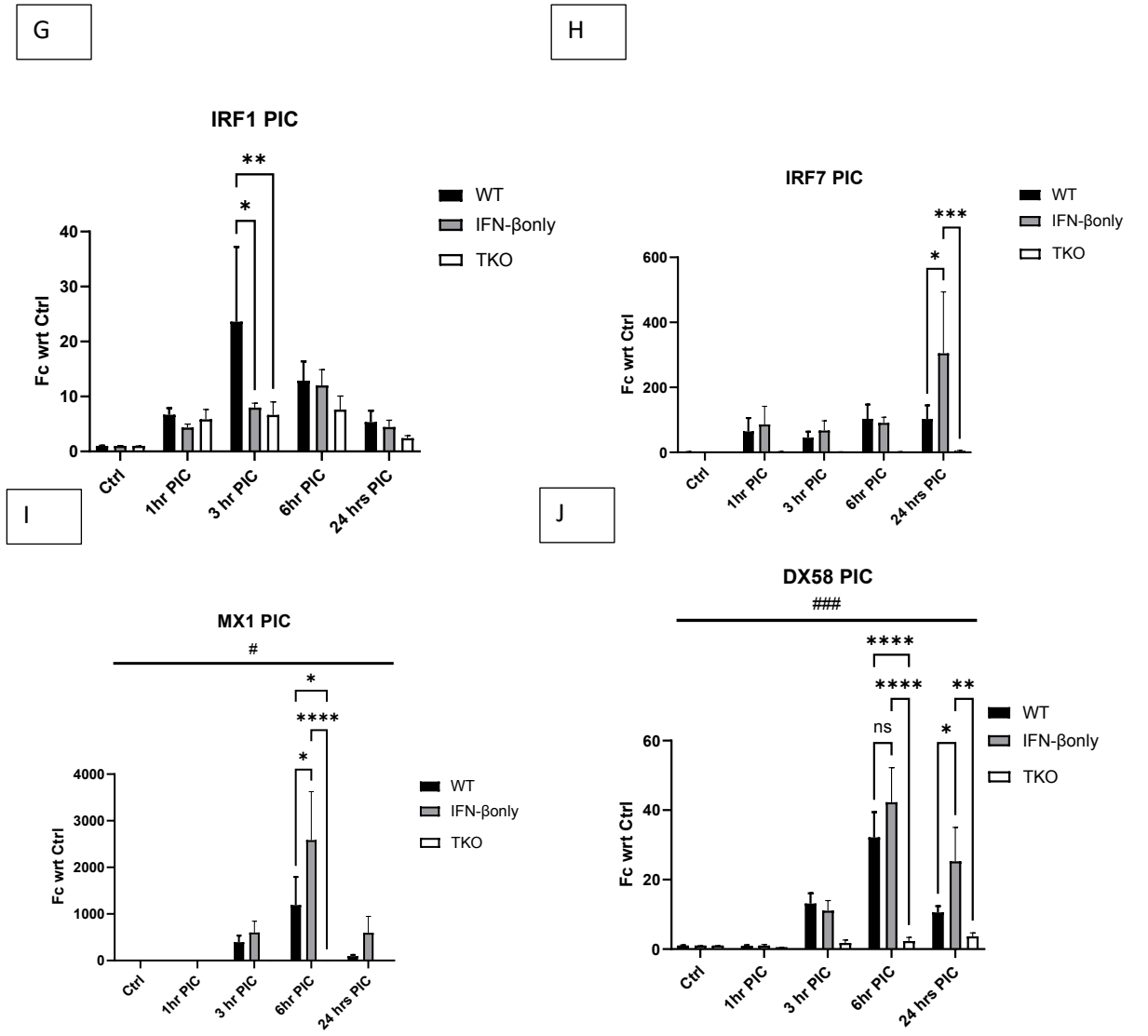


Fig. 4.6: Interferon stimulated gene expression levels in BMDMs upon PIC treatment. BMDMs from C57 BL/6 (WT), IFN-β^{only} (IFN-α KO), TKO were stimulated with 25 ug/ml PIC for the indicated times. Gene expression of interferon stimulated genes was examined using RT-PCR. Gene expression was normalized to housekeeping gene RPLP0 and Fold change wrt untreated control is presented in the data. A) Gene expression of IFN-β B) TNF-α C) ISG-15 D) Gene BST2 E) PKR F) CXCL10 G) IRF-1 H) IRF-7 I) M1XA J) DX58 in BMDMs. Each symbol represents an individual mouse (n=3 per group). Data were compared by multiple comparisons from a 2-way anova. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. # indicates the P value summary-interaction.

4.5.2.4 Gene expression levels of ISGs in BMDMs upon LPS treatment.

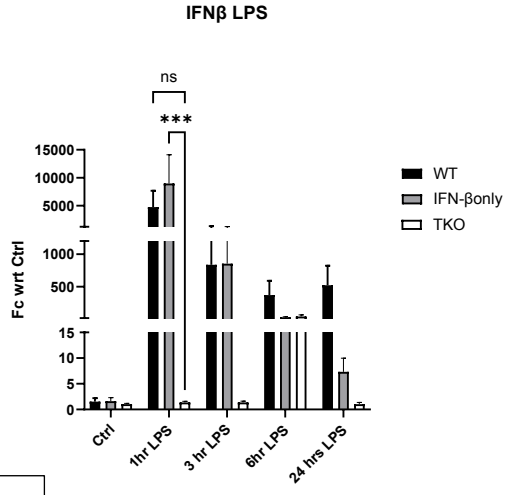
This experiment aimed to determine the gene expression level of ISGs upon LPS (100 ng/ml) induction in BMDMs using RT-PCR. Interferon stimulated genes (ISGs) measured were MX1A, ISG-15, BST2, DX58, PKR, CXCL10, IRF1, IRF7, IFN-β and the cytokine TNF-α **(Fig. 4.7).**

Here, a significant increase in ISGs, including BST2, PKR, CXCL10, IRF7 and MX1A, in IFN-β^{only} was observed when compared to WT **(Fig. 4.8 D-E, F, H-I)** TNF-α and IRF1 showed an increasing trend, but the increase was not statistically significant**(Fig. 4.8 B, G)**. ISG15 did not show any clear trend at any time point. **(Fig. 4.8 G)** Again, Supplementary figure S3 represents the same data as the previous figure but in a different format, making it easier to visualize the kinetics of the ISGs (interferon-stimulated genes).

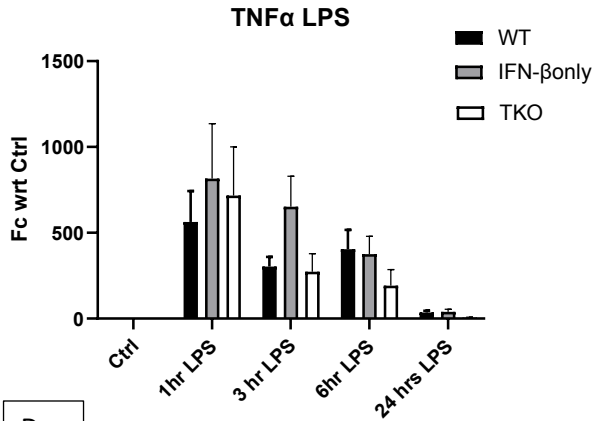
Furthermore, IFN-β or IFN-α were not detectable in TKO for LPS induction **(Fig. 4.8 A, B)**, which was expected. However, several ISGs showed expression at lower levels at various time points, like poly I: C injections. This could again indicate the influence of other signalling pathways.

Studies have shown that IFN Is play a key role in basal and stress-induced hematopoiesis.[433–440] Next, to see if there are differences in the basal vs stress hematopoiesis among these genotypes, we quantified the distinct cell populations from the bone marrow of these genotypes upon PBS, poly I:C and LPS induction.

A


IFNβ LPS
ns
***
15000
10000
5000
1000
500
15
10
5
0
Fc wrt Ctrl
WT
IFN-βonly
TKO
Ctrl
1hr LPS
3 hr LPS
6hr LPS
24 hrs LPS

B


TNFα LPS
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1000
500
0
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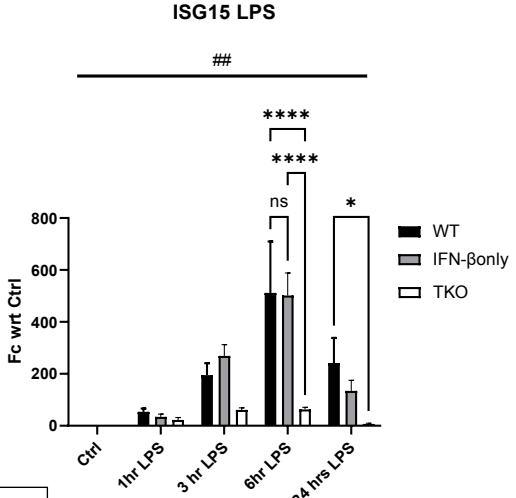
WT
IFN-βonly
TKO
Ctrl
1hr LPS
3 hr LPS
6hr LPS
24 hrs LPS

C


ISG15 LPS
##
****
****
ns
*
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600
400
200
0
Fc wrt Ctrl
WT
IFN-βonly
TKO
Ctrl
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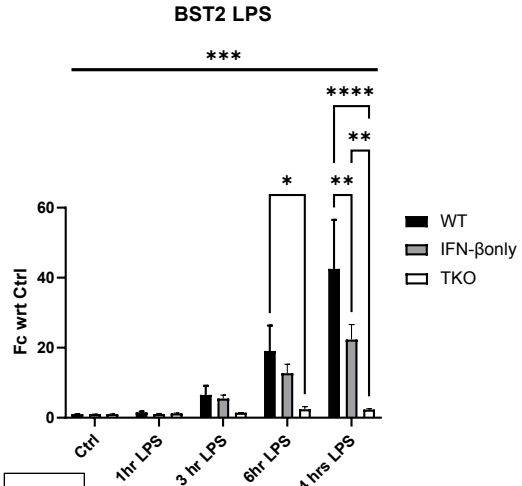
3 hr LPS
6hr LPS
24 hrs LPS

D


BST2 LPS
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****
**
*
**
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20
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Fc wrt Ctrl
WT
IFN-βonly
TKO
Ctrl
1hr LPS
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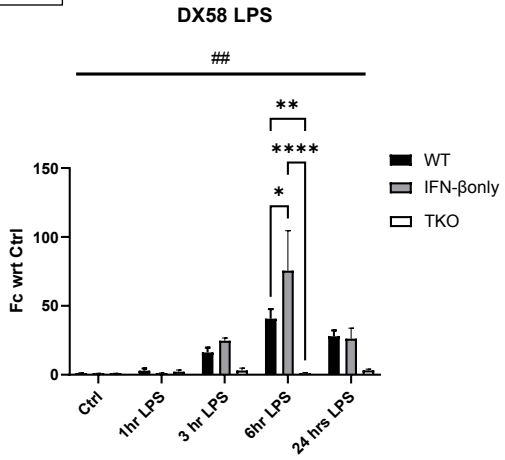
6hr LPS
24 hrs LPS

E


DX58 LPS
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**
****
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100
50
0
Fc wrt Ctrl
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IFN-βonly
TKO
Ctrl
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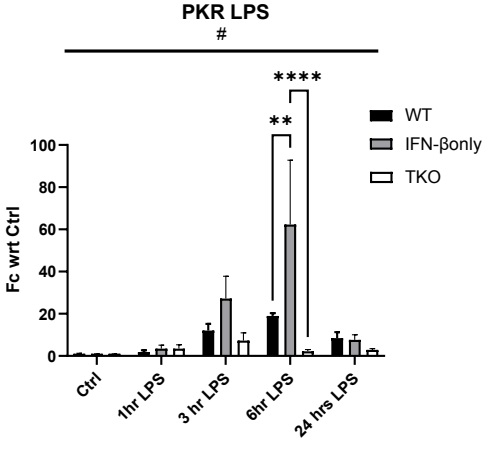
24 hrs LPS

F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****
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100
80
60
40
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WT
IFN-βonly
TKO
Ctrl
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3 hr LPS
6hr LPS
24 hrs LPS

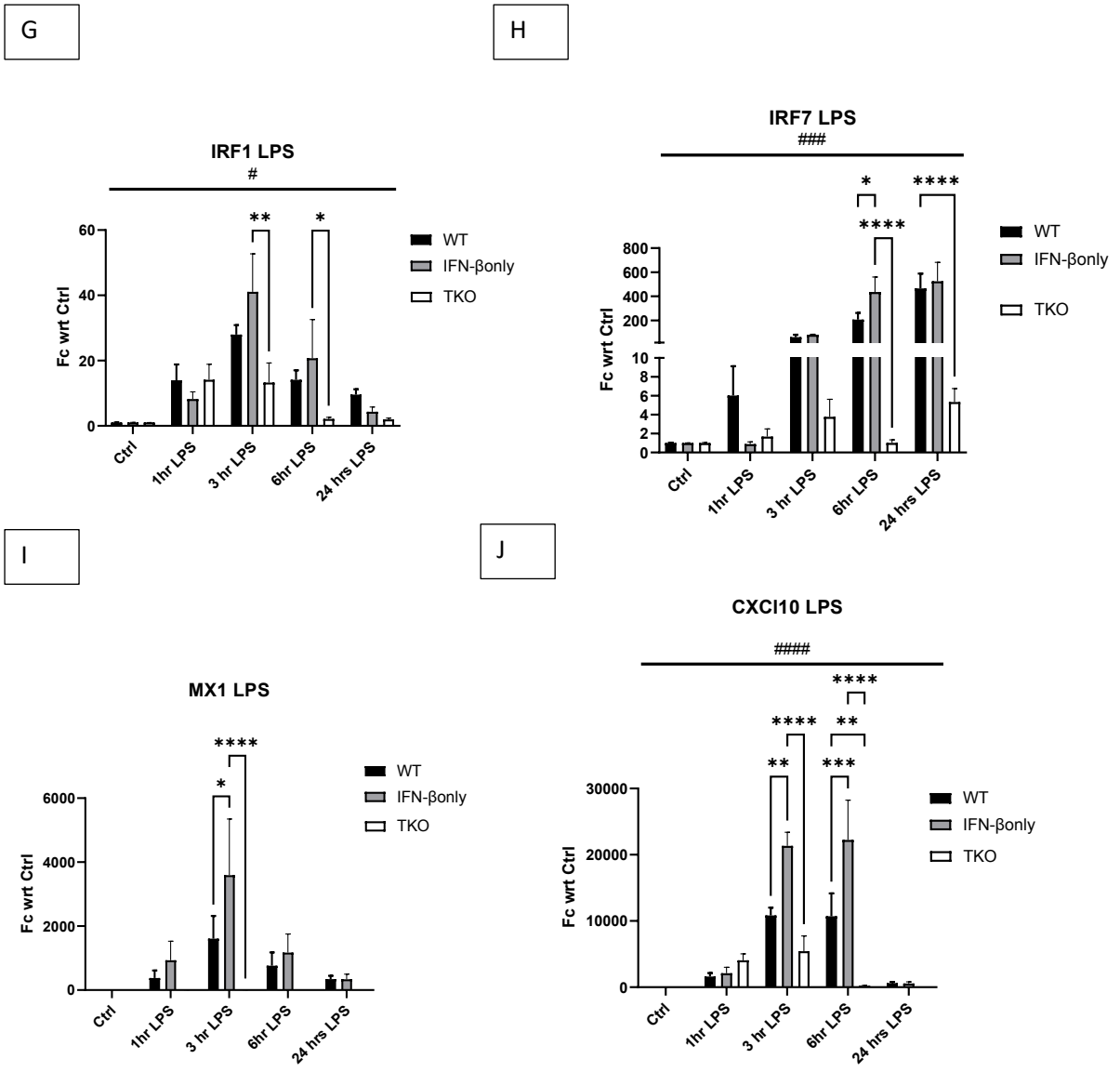

Figure. 4.7: Interferon stimulated gene expression levels in BMDMs upon LPS treatment. BMDMs from C57 BL/6 (WT), IFN-β^{only} (IFN-α KO), TKO were stimulated with 100 ng/ml LPS for the indicated times. Gene expression of interferon stimulated genes was examined using RT-PCR. Gene expression was normalized to housekeeping gene RPLP0 and Fold change wrt untreated control is presented in the data. A) Gene expression of IFN-β B) TNF-α C) ISG-15 D) BST2 E) DX58 F) PKR G) IRF-1 H) IRF-7 I) M1XA J) CXCL10. Each symbol represents an individual mouse (n=3 per group). Data were compared by multiple comparisons from a 2-way anova. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. # indicates the P value summary-interaction.

Aim III. Can IFN-β^{only} mice support basal and stress hematopoiesis?

4.5.2.5 BMDM cell composition at basal levels and upon poly I:C and LPS injection in vivo

IFN Is appear to play a key roles in both basal and stress-induced hematopoiesis, in response to infection and PAMP stimulations.[433–440] Upon Poly I:C induction, classically activated monocyte (CD11B^{+}Ly6C^{hi} cells- proinflammatory and phagocytic) was not different in IFN-β^{only} when compared to WT, but it was significantly higher in IFN-βKO **(Fig. 4.8 C)**. Similarly, CD11B^{+}Ly6C^{int} (proinflammatory) population was high in IFN-βKO than IFN-β^{only} and WT**(Fig. 4.8 F)**. Data is not shown for CD11B^{+}Ly6C^{low} cells which are alternatively activated monocytes (tissue repair), as the percentage values obtained were very lower, and can't detect significant changes. MHCII-CD11b+ cells were significantly higher in IFN-βKO than IFN-β^{only} and WT **(Fig. 4.8 G)**. T cell was significantly lower in lower in IFN-βKO **(Fig. 4.8 A).**

At basal level, MHCII+CD11b- cells were lower in IFN-β^{only} and IFN-βKO when compared to WT **(Fig. 4.8 D)** and MHCII-CD11b+ cell population was significantly higher in IFN-βKO than WT and IFN-β^{only}**(Fig. 4.8 D)**. No differences were detected for T cells, NK cells, neutrophils, and inflammatory monocytes (Ly6C^{hi}) populations**(Fig. 4.8 A, B, E, F)**. CD11B^{+}Ly6C^{int} (proinflammatory) cells were significantly lower in IFN-β^{only} when compared to WT, but higher in IFN-βKO compared to all genotypes. (Fig. 4.9). For the LPS induction, only at basal levels, Ly6Chi monocytes and MHCII-CD11b+ were significantly different among genotypes, but no conclusive data was made from this due to limited biological replicates. **(Fig. 4.9 D, E)**

All cell types except T and B cells were decreased upon stimulation compared to basal levels and all cells were comparable upon stimulation among genotypes. (Fig. 4.10) These differences in the bone marrow cell composition again indicate the differential roles of IFN-β^{only} and IFN-βKO in the process, indicating the necessity for further studies to explore more on different IFN-I subtypes.

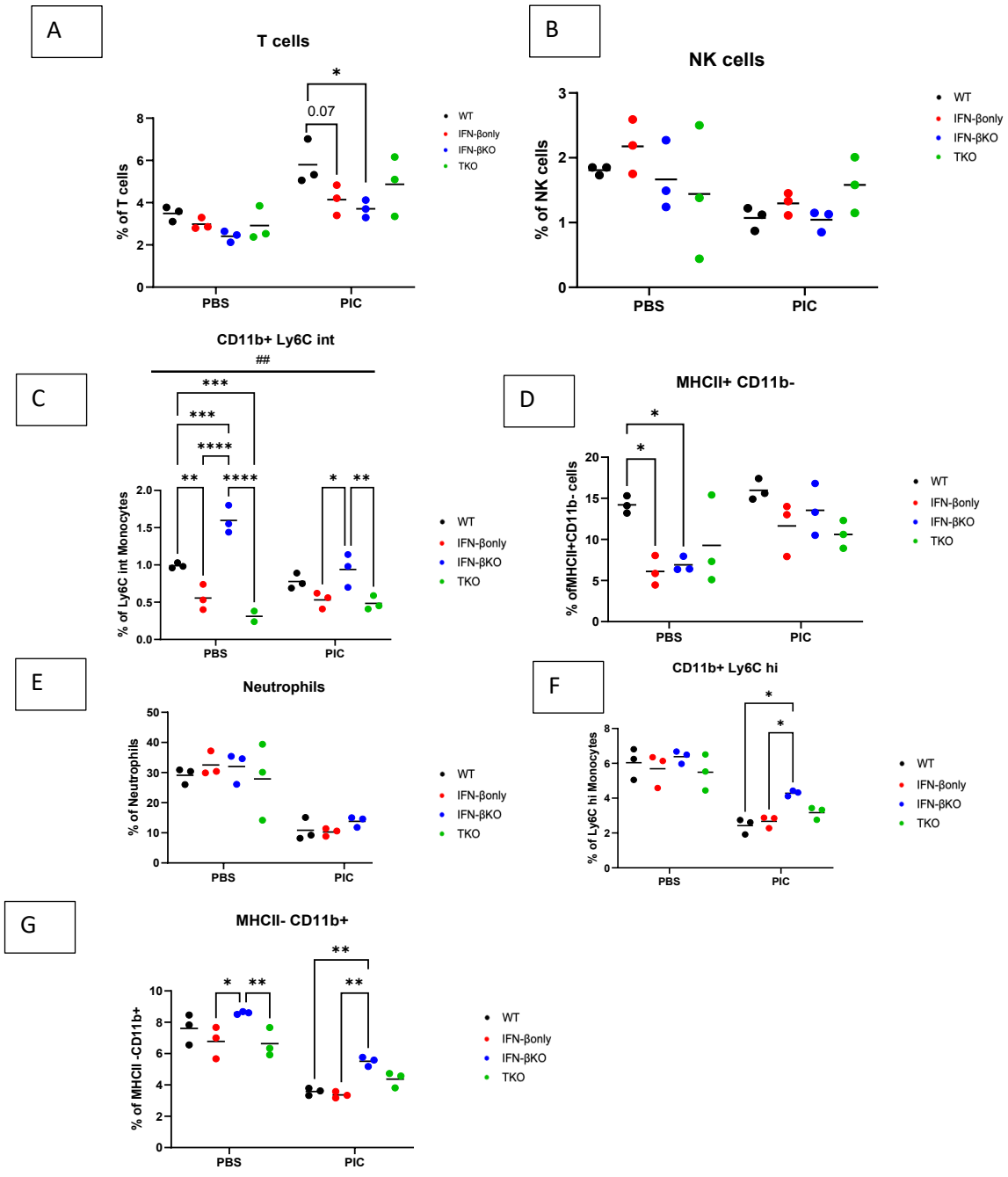

Fig. 4.8: Bone marrow cell composition of WT, IFN-β^{only}, IFN-βKO and TKO at basal level and upon poly I:C injection. C57 BL/6 (WT), IFN-β^{only} (IFN-α KO) and TKO mice were intraperitoneally injected with (200 µg) PIC and incubated the mice for 3hrs. Total bone marrow from femur was extracted and cellular composition was examined in all genotypes at basal level and upon poly I:C induction. Each symbol represents an individual mouse. A) T cells, B) NK cells, C) Ly6C intermediate monocyte D) MHC+CD11b- cells E) Neutrophils F) Ly6Ghi monocytes, F) G) MHC-CD11b+. Each symbol represents an individual mouse (n=3 per group). Data were compared by multiple comparisons from a 2-way anova. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. # indicates the P value summary-interaction.

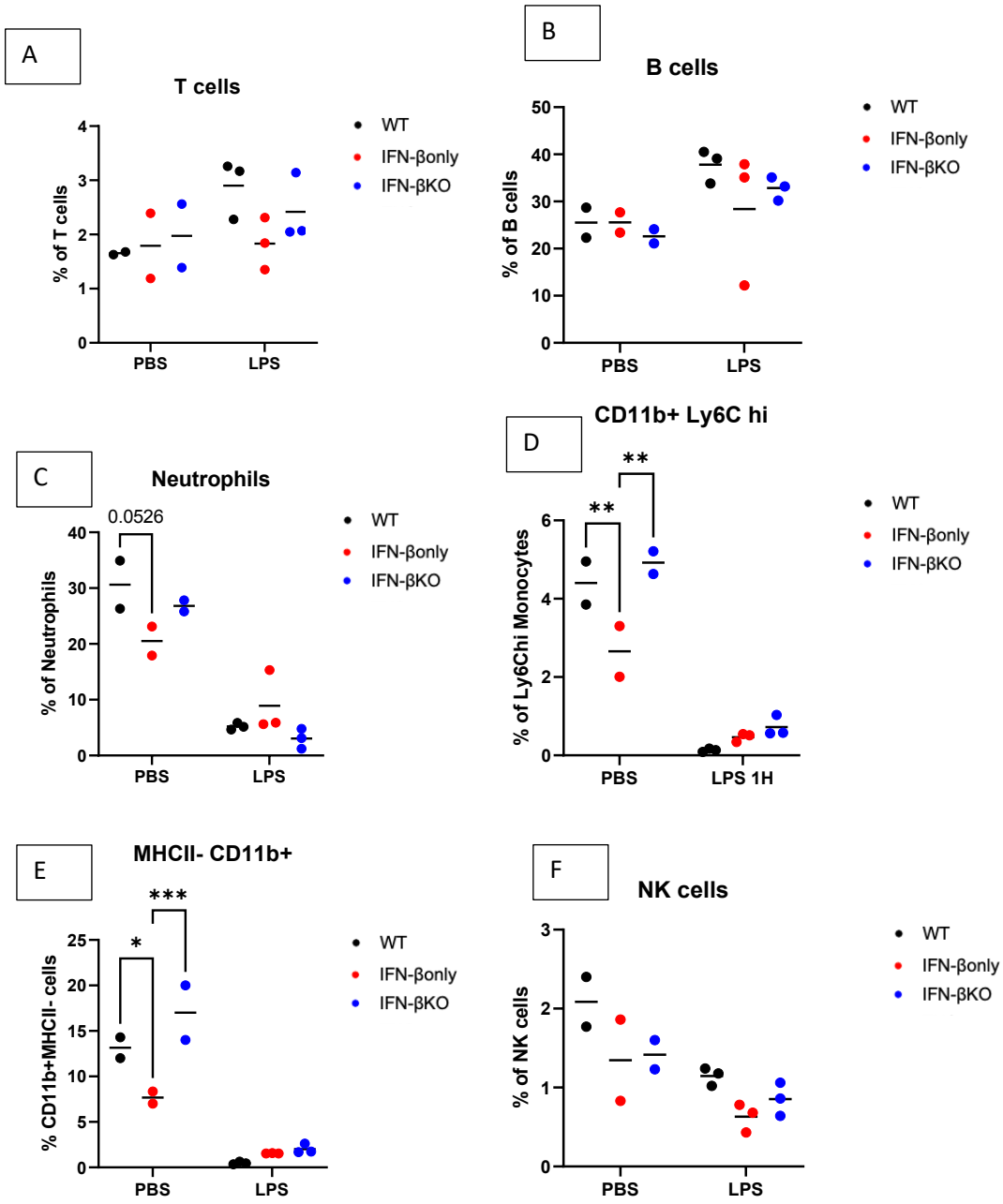

Figure. 4.9: Bone marrow cell composition of WT, IFN-β^{only} and IFN-βKO at basal level and upon LPS injection. C57 BL/6 (WT), IFN-β^{only} (IFN-α KO) and TKO mice were intraperitoneally injected with (10ug/g) LPS and incubated the mice for 3hrs. Total bone marrow from femur was extracted and cellular composition was examined in all genotypes at basal level and upon poly I:C induction. Each symbol represents individual mouse. A) T cells, B) B cells, C) Neutrophils, D) Ly6Ghi monocytes, E) MHCCD11b+ cells G) NK cells. Data are reported as means ±SEM. Stats:

2-way Anova, alpha: 0.05. (n=3) * Indicating a significant difference from multiple comparison. $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

4.6 Discussion

For the first time, this study aims to examine qualitative differences of IFN I subtypes using unique mouse models. As the first step, I employed various knockout mouse models of IFN I locus, such as IFN-β^{only} (IFN-α and other subtypes are deleted), IFN-βKO (IFN-β is deleted) and TKO (total KO), where the whole IFN-I locus is deleted (Fig. 5.1). The main question I addressed in this thesis is whether IFN-β alone (IFN-β^{only}) is enough to promote effective antiviral and systemic cytokine responses upon LCMV-Cl-13 infection as well as PAMP (poly I:C and LPS) inductions, and how that compares to IFN-β knockout and total knockout. In addition, I assessed ISG responses in BMDMs upon PAMP induction.

Following LCMV Cl13 infection, we assessed viral clearance among all genotypes. Overall, viral clearance data indicate that neither IFN-β nor IFN-αs alone are capable of controlling the virus at the early stages of infection (i.e. day 8). However, at later stages (i.e. day 60), IFN-β or IFN-αs alone appear to be enough to yield viral clearance, albeit not in all mice, as opposed to most TKO and IFNAR KO. Intriguingly, some TKO mice cleared the virus, which may indicate a yet-to-be-identified role for IFNAR in controlling viral infections.

Multiple cell types produce type I interferons as a response to various stressors, including viral infections. This response aims to induce expression of ISGs and cytokines, which coordinate IFN-I mediated antiviral, anti-tumor, inflammatory and immunomodulatory responses.[6,168,168,169,171,173,265]

Surprisingly, in vivo cytokine production in IFN-β^{only} and IFN-β KO in response to LCMV Cl-13 infection was largely intact when compared to WT, while there was a defect in serum cytokines in

TKO and IFNAR KO. This may suggest that IFN-β and IFN-αs may play redundant roles in promoting systemic cytokine production upon LCMV Cl-13 infection. However, IFN-γ level in TKO was significantly higher than all other genotypes. This is interesting because IFN-γ, a type II IFN, follows different but partially overlapping signalling pathways. Therefore, this observation could indicate that increased IFN-γ expression may be compensating for the loss of IFN Is in TKO. Decreased IFN-γ expression in IFNAR KO compared to TKO may indicate the presence of undiscovered IFNAR ligands that are not encoded in the IFN-I locus. Regardless, this assay does not account for the cellular source of these cytokines. Therefore, to assess the functional differences of T cell functions that could affect these cytokine responses, our collaborators employed flow cytometry and investigated the frequency and functionality of CD8 and CD4 T cells and other antigen-presenting cells (APCs) across the various genotypes. CD8 T cell numbers and their ability to produce cytokines (IFNγ, TNFα and IL-2) was significantly impaired in IFN-β^{only} compared to WT (data not shown).

Considering the complexity of LCMV in triggering multiple innate and adaptive immune signaling pathways, we wanted to evaluate the role of IFN-α vs β in specific innate immune signaling pathways. To this end, we used two potent pathways that activate IFN-I: LPS/TLR4 and IC: TLR3.[453–455]

Unlike following viral infection, cytokine production was altered among genotypes upon poly I:C or LPS induction. First, IFN-γ expression in TKO did not show upregulation in TKO upon poly I:C or LPS induction. In addition to inflammatory (IL-1β, IL-6, IL-12, IFN-α, IFN-β, IFN-γ, TNF-α and GM-CSF) and anti-inflammatory (IL-10) cytokines, we measured the levels of the chemokines RANTES, KC, IP-10 and MCP-1, which can stimulate and recruit immune cells to

the site of inflammation and coordinate other inflammatory responses.[456,457] Five of the thirteen assessed cytokines (IFN- α, IFN-β, IL-6, IFN-γ and MCP-1) were decreased in IFN-β^{only} compared to WT upon poly I: C induction. Nine of thirteen cytokines (IFN- α, IL-6, RANTES, IL-1β, IL-12, IL-10, IP-10, TNF-α and MCP-1) were decreased in IFN-β^{only} compared to WT upon LPS injection. This may indicate a reduced level of systemic inflammation in IFN-β^{only} mice compared to WT. Several studies have shown contrasting impacts of IFN Is on COVID-19 disease severity. For example, both robust and impaired IFN I responses are implicated to disease severity.[6,7,13,458,459] Further understanding of the roles of IFN I subtypes in inflammatory responses will provide insights in designing therapies with IFN I subtypes or inhibitors for their activities to promote balanced inflammation. Moreover, only the chemokine KC (CXCL1) showed a significant increase in IFN-β^{only} when compared to IFNβKO. KC (CXCL1) is a proinflammatory chemokine shown to recruit immune cells mainly neutrophils to the site of inflammation.[460–462] Overall, the observed PAMPs induced differential cytokine responses among genotypes varied from virus induced responses which results from the activation of multiple signaling pathways.

Macrophages are crucial in producing IFN Is and are essential for the immune system's response to infection and inflammation.[455] Bone marrow-derived monocytes rapidly contribute to IFN-I production in most inflamed tissues during stress.[463] Thus, macrophages are excellent cell types to examine IFN I induced ISG signatures. In this study, we again used poly I:C and LPS to stimulate the macrophages in vitro and study the differences in the responses between IFNβ and IFNαs.

Following stimulation with LPS or Poly I:C, five (IFN-β, IRF7, PKR, DX58 and MX1) of the ten measured ISGs were significantly increased in the absence of IFN-α (in IFN-β^{only}) cells compared

to WT, while one (IRF1) was decreased. IFR7 and IRF1 are transcription factors critical for IFN I expression.[464] Protein kinase R (PKR) is first identified as an antiviral factor, but is now known to be involved in multiple processes including transcriptional control, mRNA translation, cell proliferation and regulation of apoptosis.[465,466] DX58, also known as RIG I, is a cytosolic PRR that recognizes viral RNA.[467] Increase in these ISGs upon lacking IFN-α coupled with increased IFN-β expression in BMDMs may indicate a role for IFN-β in their expression. MX1 is shown to be remarkably reduced upon lacking IFN I receptor (IFNAR) or IFN-β, though they can also be induced by type III interferons.[1–3] In contrast, IFN-α deletion has shown increased MX1 expression levels in our data. Interestingly, considering the ISGs that involve in viral life cycle, MX1 is shown to restrict viral transcription and replication during uncoating, and PKR is shown to restrict viral protein translation. Interestingly, absence of both IFN-α and IFN-β in TKO markedly reduced all ISG expression levels. Overall, our data on ISG expressions in BMDMs indicating an immunomodulatory role for IFN-α as its absence increases the above discussed ISGs. BST2 was significantly decreased in the absence of IFN-α upon LPS induction but not in poly I:C induction. This could suggest a cross talk between NF-κB signaling and Type I interferon signaling during LPS stimulation. Future studies comparing these responses to IFN-β and other subtypes including individual α subtypes will improve our understanding on this question. Thus, this analysis on primary BMDMs allowed us to examine the macrophage specific IFN I responses. Studying the responses in different cell types is essential as the IFN I subtypes can have cell type specificity, as shown for IFN-ε and IFN-κ.[202,204–206,291,292] Furthermore, unlike mRNA levels in BMDMs, serum cytokine levels represent their net release from various cell types. Several cell types, including other immune cells, fibroblasts, and epithelial cells, can produce IFN Is and ISGs.[470] Thus, the differences in serum cytokine levels and ISG levels in BMDMs together validate

the previous observation that IFN-β and IFN-α subtypes could possess qualitative functional differences in their responses.

Lower IFN-α and IFN-β levels detected in their respective knockouts in the Legendplex™ Mouse Anti-Virus Response Panel assessment upon PIC and LPS injection, but not in TKO might be due to the lower assay specificity between IFN- a subtypes and IFN- β. IFN-a subtypes in humans have 80% homology and the other subtypes share 30-50% homology.[172] IFN-ε, a protein of 192 amino acids, shares around 30% similarity with IFN-α and IFN-β in humans.[471,472] IFN-ζ also shows high nucleotide homology with IFN-β and IFN-α at many residues.[451,452] This suggests that the antibodies in the assay may have non-specific binding between IFN-β and IFN-α.

Furthermore, altered bone marrow cell composition was observed among genotypes. Mainly, significant differences among genotypes were observed in myeloid cells (CD11B+ cells). Upon Poly I:C induction classically activated monocyte ($CD11B^{+}Ly6C^{hi}$ cells- proinflammatory and phagocytic) was not different in IFN-β^{only} when compared to WT, but it was significantly higher in IFN-βKO. Similarly, $CD11B^{+}Ly6C^{int}$ (proinflammatory) population was high in IFN-βKO than in IFN-β^{only} and WT at resting state and upon Poly IC induction. Overall, this increased Ly6C expressing monocytes may suggest a role in inflammatory monocyte differentiation during hematopoiesis.[456,475]Data is not shown for $CD11B^{+}Ly6C^{low}$ cells which are alternatively activated monocytes (tissue repair), as the percentage values obtained were very lower, and can't detect significant changes. Furthermore, higher MHCII-CD11b+ cells (granulocytes such as eosinophils gated out from neutrophils and NK cells) in IFN-βKO compared to IFN-β^{only} and WT upon poly I:C induction and at resting state, lower MHCII+CD11b- cells in IFN-β^{only} and IFN-βKO when compared to WT and MHCII-CD11b+ cell population was significantly higher in IFN-βKO than

WT and IFN-β^{only} at resting state also suggest differential regulation of hematopoiesis by IFN-α and β.[433,434,476] Altered ISGs and cytokine levels such as BST2 and GM-CSF that are known to modulate bone marrow hematopoiesis could explain these differences.[476]

Finally, dysregulated IFN-I responses are linked to several pathologies, including chronic infections, cancer, and autoimmune diseases. In line with previous studies which showed qualitative roles for certain IFN I subtypes[175], our study showed that both IFN-α and IFN-β are essential to control early viral dissemination as WT and there is a specific role for IFN-α in controlling early viral dissemination. Furthermore, ISG and cytokine signatures as well as hematopoiesis upon LPS and poly I:C induction showed subtype specific differences. Taken together, these findings suggest that the IFN-I subtypes have unique roles in antiviral and inflammatory responses. To further study the individual IFN-α subtypes in this context, we will knock in particular IFN-I subtypes individually into these KO mice in the future.

5 OVERALL SUMMARY OF THE FINDINGS AND FUTURE DIRECTIONS.

Overall, our first study demonstrated how chronic moderate exercise can cause persistent epigenetic, metabolic, and functional adaptations in macrophages, which influence their inflammatory responses. Future studies should focus on how chronic moderate exercise affects disease progression and outcome in in vivo models of autoimmune disease or infection. Further, examining the persistent immunometabolic adaptations in immune cells from various other tissues such as muscles, blood immune cells, lungs, etc. will provide in-depth understanding. The second approach, which employed genetic manipulation of IFN-Is, suggest that IFN-I subtypes have unique and non-redundant roles in immune responses. Specifically, IFN-α was vital in early viral clearance and cytokine and ISG responses upon viral infections and PAMPs inductions. This

suggests a potential therapeutic target to regulate infection-related inflammation while encouraging the need to study the individual subtypes using reliable in vivo models. To further study the individual IFN-α subtypes in this context, we will knock in specific IFN-α subtypes into the TKO mice and examine their specific roles in antiviral and inflammatory responses in mice. Overall, data from both approaches contributed significantly to our understanding of the mechanisms behind exercise adaptations and IFN I subtype mediated inflammation regulation.

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7 APPENDIX A: Additional figures and tables

Table S1 Primer sequences Chapter 4

Primer name	**Forward 5' to 3'**	**Reverse 5' to 3'**
RPLP0	GGACCCGAGAAGACCTCCTT	CAACTCGCTCCAAGA TTC
IL-10	ATAACTGCACCCACTTCCCA	TGAGGACA TCTCCCACGTCAA
IL-1β	GCAGCACATCAACAAGAG	CAGCAGGTTATCATC- ATCATC
TNF-α	AGAATGAGGCTGGATAAGAT	GAGGCAACAAGGTAGAGA
TGF-β	CCTGCAAGACCATCGACATG	TGTTGTACAAAGCGAGCACC
IFN-β	TCCAAGAAAGACGAACA TTCG	TGAGGACA TCTCCCACGTCAA
iNOS	ATAACTGCACCCACTTCCCA	GGGCATCACTTCTACCAGGT
Hif1- α	AGCTTCTGTTATGAGGCTCACC	TGACTTGATGTTCATCGTCCTC
Irg	GCAACATGATGCTCAAGTCTG	TGCTCCTCCGAATGATACCA
Hmox-1	CGCCTTCCTGCTCAACAT	ACGAAGTGACGCCATCTG
Arginase-1	GATTATCGGAGCGCCTTTCT	TGGTCTCTCACGTCATACTCT
uPKF2	GTTCTACGCTGCCTACTA	TGCTCACCGATTCTACAT

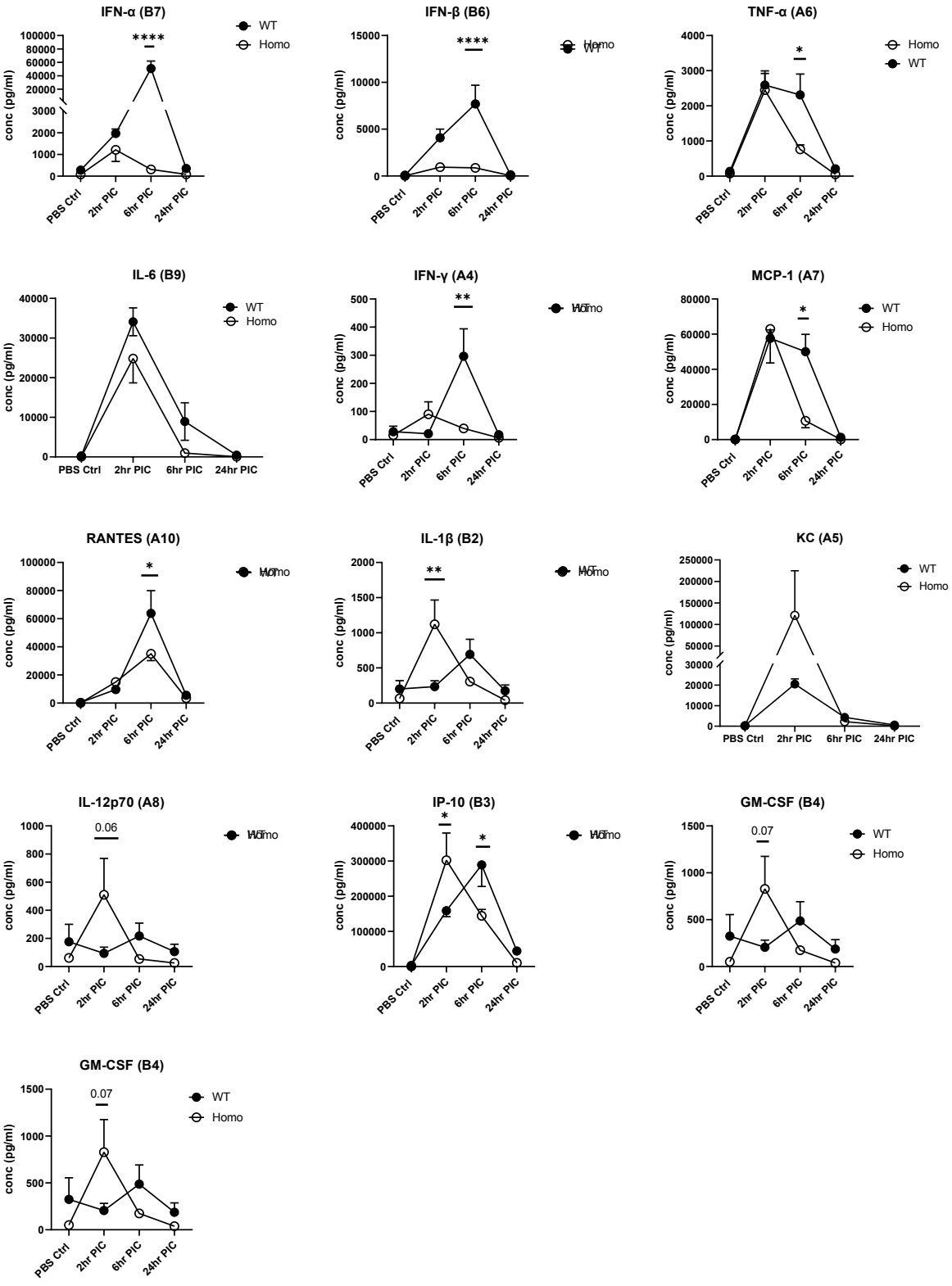

IFN-α (B7)
IFN-β (B6)
TNF-α (A6)
IL-6 (B9)
IFN-γ (A4)
MCP-1 (A7)
RANTES (A10)
IL-1β (B2)
KC (A5)
IL-12p70 (A8)
IP-10 (B3)
GM-CSF (B4)
GM-CSF (B4)
conc (pg/ml)
WT
Homo
PBS Ctrl
2hr PIC
6hr PIC
24hr PIC
****
**
*
0.06
0.07

Fig. S1: Interferon stimulated gene (ISG) levels in serum upon PIC IP injection-time course. C57 BL/6 (WT), IFN-β^{only} (IFN-α KO) mice were intraperitoneally injected with (200 ug) PIC and incubated the mice for indicated times. Serum levels of cytokines were examined via flow cytometry-based Elisa experiment (legend plex assays). A) Serum levels of IFN-α B) Serum levels of IFN-β C) Serum levels of IL-6, D) Serum levels of IFN-γ, E) Serum levels of RANTES F) Serum levels of IL-1β G) Serum levels of IL-12. H) Serum levels of IP-10, I) Serum levels of IL-10, J) Serum levels of TNF-α, K) Serum levels of MCP-1, L) Serum levels of KC, M) Serum levels of GMCSF Each symbol represents individual mouse. Data are reported as means ±SEM. Stats: 2-way Anova, alpha: 0.05. (n=3) * indicating a significant difference from multiple comparison. p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

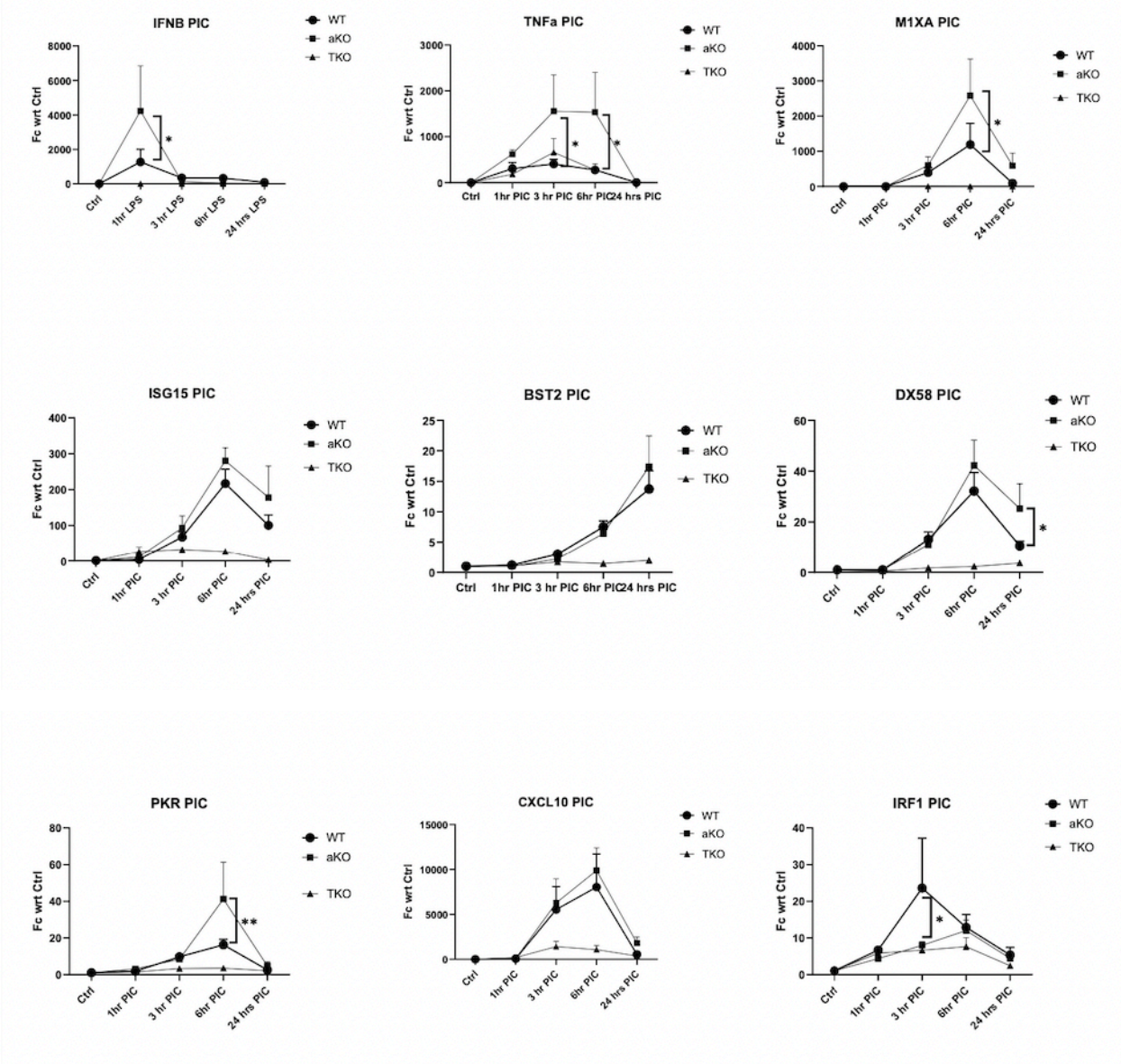
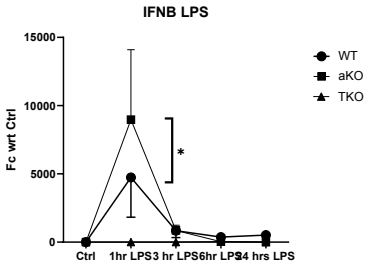


Figure. S2: Interferon stimulated gene expression levels in BMDMs upon PIC treatment-time course BMDMs from C57 BL/6 (WT), IFN-β^{only} (IFN-α KO), TKO) were stimulated with 100 ng/ml LPS for the indicated times. Gene expression of interferon stimulated genes were examined using RT-PCR. Gene expression was normalized to housekeeping gene RPLP0 and Fold change wrt untreated control are presented in the data. A) Gene expression of IFN-β B) Gene expression of TNF-α C) Gene expression of ISG-15 D) Gene expression of BST2 E) Gene expression of PKR F) Gene expression of CXCL10 G) Gene expression of IRF-1 H) Gene expression of IRF-7 I) Gene expression of M1XA J) Gene expression of DX58 K) Each symbol represents individual mouse. Data are reported as means ±SEM. Stats: 2-way Anova, alpha: 0.05. (n=3) * indicating a significant difference from multiple comparison. $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.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10000
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Ctrl
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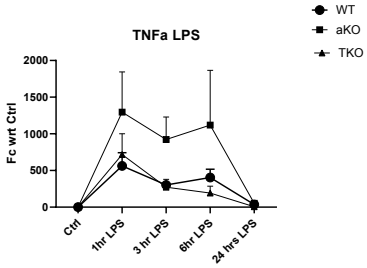
3 hr LPS
6hr LPS
24 hrs LPS
WT
aKO
TKO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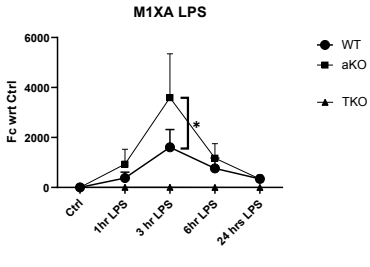
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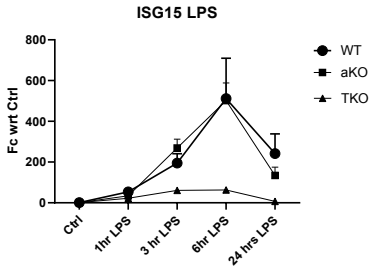
3 hr LPS
6hr LPS
24 hrs LPS
WT
aKO
TKO


ISG15 LPS
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1hr LPS
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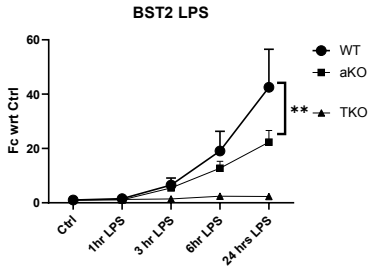
BST2 LPS
Fc wrt Ctrl
60
40
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0
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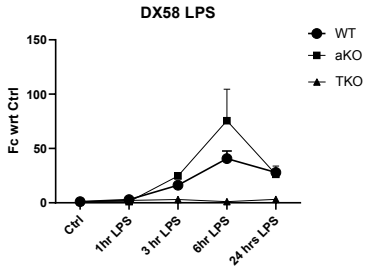
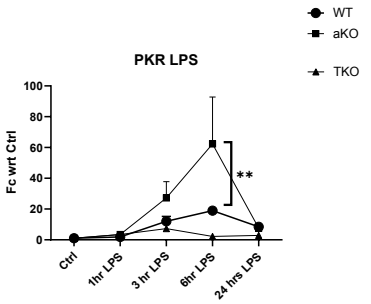
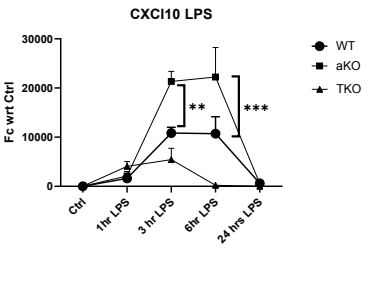
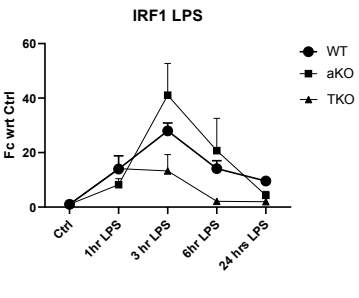
Ctrl
1hr LPS
3 hr LPS
6hr LPS
24 hrs LPS
WT
aKO
TKO


DX58 LPS
Fc wrt Ctrl
150
100
50
0
Ctrl
1hr LPS
3 hr LPS
6hr LPS
24 hrs LPS
WT
aKO
TKO


PKR LPS
Fc wrt Ctrl
100
80
60
40
20
0
**
Ctrl
1hr LPS
3 hr LPS
6hr LPS
24 hrs LPS
WT
aKO
TKO


CXCl10 LPS
Fc wrt Ctrl
30000
20000
10000
0
**
***
Ctrl
1hr LPS
3 hr LPS
6hr LPS
24 hrs LPS
WT
aKO
TKO


IRF1 LPS
Fc wrt Ctrl
60
40
20
0
Ctrl
1hr LPS
3 hr LPS
6hr LPS
24 hrs LPS
WT
aKO
TKO

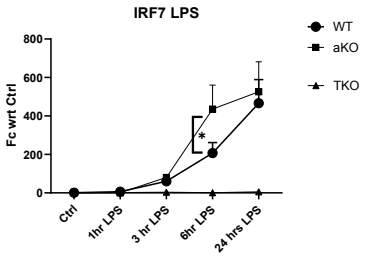

IRF7 LPS
Fc wrt Ctrl
800
600
400
200
0
*
Ctrl
1hr LPS
3 hr LPS
6hr LPS
24 hrs LPS
WT
aKO
TKO

Fig. S3: Interferon stimulated gene expression levels in BMDMs upon LPS treatment. BMDMs from C57 BL/6 (WT), IFN-β^{only} (IFN-α KO), TKO) were stimulated with 100 ng/ml LPS for the indicated times. Gene expression of interferon stimulated genes were examined using RT-PCR. Gene expression was normalized to housekeeping gene RPLP0 and Fold change wrt untreated control are presented in the data. A) Gene expression of IFN-β B) Gene expression of TNF-α C) Gene expression of ISG-15 D) Gene expression of BST2 E) Gene expression of PKR F) Gene expression of CXCL10 G) Gene expression of IRF-1 H) Gene expression of IRF-7 I) Gene expression of M1XA J) Gene expression of DX58 K) Each symbol represents individual mouse. Data are reported as means ±SEM. Stats: 2-way Anova, alpha: 0.05. (n=3) * indicating a significant difference from multiple comparison. $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

Table S2: Antibodies used in spectral flow experiments.

Fluorophores	**Fluorophores-Channel**	**Dilution factors**
CD3	ef450-V3	1:50
NK1.1	SB702-V13	1:50
Ly6G	SB600-V10	1:50
CCR2	APC-R1	1:50
MHCII	AF700-R4	1:800
Ly6C	AF488-B2	1:400
CD11B	SB645-V11	1:400
LD	LD Blue-V6	1:200
CX3CR1	BV 785-V15	1:1000
CD19	PE-YG-1	1:800
p-S6	Alexa Flour TM 488	1:50
P-P38	PE	1:50
PErk	Alexa Flour TM 647	1:50

8 APPENDIX B: Experimental methods

8.1 Dissection, bone marrow flushing, plating.

Dissection and bone marrow flushing procedure (per 1 mouse) and treatments.

Materials necessary for one mouse

S+ (about 60 ml), LCM 8 ml-20oC), 10 ml Syringe (1), 27-gauge needle (1), 23-gauge needle (1), Bacterial cell culture petri dishes (8), Tissue culture petri dish to keep the bones in S+ (1)

Day 0: Dissection, bone marrow flushing, plating.

1) Clip the mouse abdomen facing upward in a Styrofoam board using needles.
2) Spray 70% ethanol to the abdomen and the hind legs of the mouse.
3) Make an incision in the middle of the abdomen and dissect the skin from the abdomen and legs. Clean the muscle attaching the hind legs and the pelvis. Remove the leg by cutting right after the joint in the pelvis, but close to the joint. (Make sure you don't cut in the leg, that opens the bone marrow to the air).
4) Cleanup the extra muscle from the femur and tibia using scissors and forceps. Use 70% ethanol to wet the bones at any point before you open the bone marrow. Keep the bones in S+ media in a 6 cm dish or 15 ml conical tube.
5) Separate the tibia and femur by cutting at the cartilages between them**.** Cut the bones right after the joint to open the bone marrow.
6) Flush each bone with 2.5 ml S+ media from both sides (2ml from one side and 0.5) ml from the other side) and collect in a 50 ml conical tube (10 ml per mouse).

7) Centrifuge all tubes at 1500 rpm at 4°C for 5 min and throw the supernatant in waste in the BSC.
8) Resuspend the pellet in 2 ml Red blood cell Lysis buffer by pipetting up and down and remove the cell debris by tilting the tube and remove it by the pipette tip. Incubate it for 5 min at RT and after the incubation and add 5 ml S+ buffer and centrifuge it at 1500 rpm at 4°C for 5 min.
9) Discard the supernatant and resuspend the pellet in 40 ml S+ media containing 25% LCM by pipetting up and down and plate 5 ml of cells mixture in bacterial cell culture Petri dishes and let the cells grow for three days.
10) Incubate it for 3 days in the 37°C, 5% CO2 incubator.

Day 3: Scraping the macrophages from Bacterial cell culture plates

1) Discard the media and add 3 ml of S+ media on top of them.
2) Scrape the cells in one direction and transfer them in a conical tube (24 ml per mouse).
3) Count the cells under the microscope using the hemocytometer. Use trypan blue dye 10µl+10µl of your scraped cells mixture. Mix it in one well of 96 well U/V bottom plate. And load it in the Hemocytometer, make sure it covers the total grid.

Seed $5x10^5$ cells in S+ media containing 25% LCM media in 6 well plates and let them proliferate and differentiate for 2 more days. At this point, you get M0 Macrophages.

S+ media

500 ml RPMI broth

50 ml FBS (thaw one conical tube from -80°C)

5.5 ml GPPS (Stock is 100X)

5.5 ml NEAA (100X)

550 ml 2-ME for cell culture (1000X)

For treatment (in this case, M1/M2 polarization)

1) Discard the media and wash the cells with ice cold 1X PBS without Ca^{2+} and Mg^{2+}.
2) Add 1 ml of activation media to each well.
3) Incubate it for 24 hrs at 37°C in CO_2 incubator.
4) Discard the media and wash the cells with ice cold 1X PBS containing Ca^{2+} and Mg^{2+}.
5) Add 1ml trizol in the fume hood and incubate it for 5 min at RT.
6) Scrape the plate with 1ml pipette tip bottom and transfer it into an eppendorf tube and do the RNA extraction or store at -80°C and do the RNA extraction later.

Activation media for M1 and M2 Macrophages (for 10 ml)

Components	**M1**	**M2**	**Stock**
S+	**10ml**	**10 ml**	
Gentamycin	**4ul**	**4ul**	**50 mg/ml**
LPS	**10ul**	**-**	**100 ug/ml**
Ifnγ	**2ul**	**-**	**100 ug/ml**
IL4	**-**	**2ul**	**100 ug/ml**

Pineda-Torra et al. (2015)

Activation medium M1: 10 % FBS-LE, 20 μg/mL gentamycin,

20 ng/mL IFNγ, 100 ng/mL of LPS.

Activation medium M2: 10 % FBS-LE, 20 μg/mL gentamycin,

20 ng/mL IL-4.

For Treatments other than polarization

1. Cells should be plated in tissue culture dishes with desired cell numbers and volume.

2. After Day 5, cells should be washed with PBS with No Ca and Mg and add fresh media.
3. Treat the cells with desired treatment conditions.

8.2 RNA extraction from cells in the plate

Treating with Trizol

1. Wash the cells on the plates with ice cold 1X PBS with Ca^{2+} and Mg^{2+}
2. Add 1 ml (per condition) Trizol reagent in the fume hood.
3. Incubate at RT for 5 min.
4. Scrape with the bottom of the 1 ml pipette tip, collect in eppendrof tubes and store at -80^{o}C until RNA extraction.

Extraction

1. Clean everything gloves, tip boxes, pipettes with RNAase Zap to remove RNAse from surfaces.
2. Add 200 µl of chloroform to each sample.
    a. *When you take chloroform in pipette- pipette up and down to avoid dripping*
3. Vortex for 15 sec
    a. *Solution will turn milky, and aqueous layer will become upper layer.*
4. Centrifuge for 15 min 12500 rpm at 4^{o}C
    a. *If you have more sample to put in two sets, start removing the aq layer when the 1st set is done.*
5. Remove total 400 µl, 200 µl at a time when you remove aq layer which has RNA and add into a new eppendrof tube.
    a. *If you disturbed the bottom layer in the second time avoid the second 200* µl.
6. Add 500 µl isopropanol to 400 µl aq layer that was separated and mix by inverting gently.

7. Incubate 10 min at RT.
8. Centrifuge at 12 000 rpm for 10 min at 4°C.
9. Aspirate the supernatant using Vacuum.
10. Add 1 ml 70% ethanol made in MBG water and mix gently by tapping.
11. Centrifuge 10 000 rpm for 5 min.
12. Aspirate the ethanol → leave about 50 µl ethanol at the bottom and remove it by 200 µl pipette tip.
    a. *Check the tip when you remove ethanol. If you see pellet moved into the tip, put it back and spin it and remove the ethanol again.*
13. Remove the ethanol as much as possible, otherwise it takes long time to try the tubes.
    a. *Before you let it dry, note down the approximate water volume you are you are going to dissolve in. (20 to 30* µl*)*
14. Dissolve the RNA in appropriate volume (20 to 30ul) and store at -80°C.
    a. *You can freeze and thaw only once from -80°C. Its better to do the RNA conc measurement and reverse transcription on the same day, if possible.*

8.3 Surface staining protocol for flow-cytometry experiment

1. Bone marrow cells were collected in the falcon tubes and centrifuge for 5 min at 4°C at 1500 rpm.
2. Resuspend the pellet in the 2 ml red blood cell lysis buffer (sigma) and incubate at RT for 5 minutes. Add 5 ml of FACS buffer (1X PBS with 2 % FBS) on top of RBC ysis buffer and centrifuge at 1500 rpm for 5 minutes.
3. Transfer the cell suspension to 96 well plates (based on the cell numbers and number of wells you need)

4. Cells were then spun down at 1500 rpm for 2 min at 4°C (with FACS buffer-washing)
5. Resuspend pellet in 200ul diluted FC block (1:100 dilution) in FACS buffer and incubated on ice for 10 min.
6. Wash the cells with FACS biffer 2 times.
7. ***From the following step, Protect the cells from light in all incubation.***
8. Centrifuge and resuspend in 50ul Antibody mixture (Surface stain mix)
9. **Prepare the antibody surface based on the dilution factors of antibodies and total volume you need (Refer to table S2)**
10. If the total number of well is 10 (10 *50ul) → 500 ul
11. Mix and incubate the cell antibody mix at 4°C for 30 min (protected from light)
12. Add 200 ul FACS buffer on top and centrifuged at 1500 rpm 4°C for 2 min.
13. Resuspend the cell pellet in 200 ul FACS buffer and centrifuge. (washing)
14. If you need to fix and store the cells, resuspend the washed pellet in1 X IC fixation buffer and incubate at RT for 30 min (Protected from light)
15. Centrifuge and resuspend in 200ul FACS buffer and store at 4°C (mix before analysing the samples).

    For intracellular Phospho- staining, see the protocol "phospho flow staining protocol."
16. For Reference samples/compensation samples, cells were single stained with antibodies and stains and loaded to the plates. Unstained samples were prepared for each different mouse types and treatments.
17. Analyse on Attune or Cytec spectral flow-cytometry. (Refer to the next section)

8.4 SOP spectral flow experiment

Instrument start-up and daily QC (performance test)

Turn on the instrument and the plate reader. (Make sure the supply water contains enough ddH2O and the waste bottle is empty before you start)

Turn on the spectral flow program in the computer and log in to your user account. When you create user account make sure you select “bypass instrument warm-up”.

Instrument will take 30 minutes warm-up time; run MPG water for 30 minutes (min 15 minutes) at high speed.

After the warmup is done, run daily QC; Vortex the QC beads well and add one drop to 300 ul of MBG water and run QC. Check for single clean peaks on each histogram during the run and for the green check mark at the end of the assay.

- Create new experiment.
- Run reference control and Un-mix.
- Run samples.
- Check nxn. Plots and export FCS files, and analyse the unmixed files.

8.5 Treatment and intracellular staining procedure for inflammatory-metabolism markers

1. Plate the cells in the appropriate media
2. When the cells are ready for the treatment
    a. Wash with DPBS
    b. Change the media to plain RPMI (1ml)
3. Incubate in plain RPMI for 2 hrs (Serum starving)
4. After 2 hrs of serum staving, change to complete media (e.g., S+)

5. Treat the cells with appropriate treatments
6. At the end of each treatment, remove the media and immediately add 200ul of fixation
7. buffer into the wells for adherent cells
8. *For the suspension cells, pellet the cells and then add the fixation buffer in the tubes.*
9. Incubate the cells in fixation buffer for 30 min at RT. (towards the end of the fixation time
10. start scraping the cells and collect in to round bottom 96 well plates.)
11. Centrifuge the plate at 1200 rpm for 2 min and add ice cold methanol 150 ul (methanol permeabilization) and incubate at 4^{o} C at least for 20 min and proceed with intracellular cells staining (see the procedure for intracellular cells staining/ phospho staining)
12. Add 100 µl FACS buffer on top, centrifuge and resuspend the cells in FACS buffer.
13. Wash the pellet one more time in 200 µl FACS buffer (centrifuge at 1200 rpm 4^{o}C for 2 min).

Intracellular staining

14. *For antibody mix preparation refer to the file "Ab fluorophore flow dilution factors. (Table S2)"
15. Resuspend the pellet in intracellular antibody mix 50ul and incubate at 4^{o}C for 25 min (Antibody mix is prepared in FACS buffer) protected from the light.
16. Add 200 µl FACS buffer on top, centrifuge 1200 rpm for 2 minutes and repeat this washing one more time in 200 µl FACS buffer and then the cells are ready to be run in the Flow cytometry.
17. *Fluorochromes of intracellular antibodies should be chosen carefully-Check if they are methanol resistant.*

18. ***Prepare 50 ul of FMOs and single stained compensation samples for each different antibody. Cell sample for FMOs should be used from treated cells.**

8.6 MitosoX and MBBr and JC-1 staining.

1. Trypsinise/scrape the cells and take it in to 15 mil tube and centrifuge at 300g for 5 min.
2. Resuspend the pellet in desired volume of FACS buffer and transfer to 96 well plates.
3. Centrifuge the plate at 1200 rpm for 2 min at 4^{o}C.
4. Resuspend the cells in 200 ul of MitosoX and MBBr stain mix prepared as shown below.
5. Incubate for 10 min at 37^{o}C in the incubator.
6. Centrifuge and resuspend the cells in 200 ul FACS buffer.
7. Run the assay.

Preparing the Stain mixture in the FACS buffer

For Mitosox and MBBr assay

Mitosox (5 mM stock) → 0.5 ul in 1 ml FACS buffer (Final Conc 2.5 uM)

MBBr (40 mM stock) → 1 ul in 1 ml FACS buffer (Final conc 40 uM)

Live dead (1: 800 dilution) 1.25 ul in 1 ml FACS buffer (optional- depending on the experiment)

For JC-1 assay

JC-1 (2.5 mM stock) → 2 ul in 1 ml FACS buffer (Final conc 5 uM)

JC-1 2 ul in 1 ml FACS buffer

8.7 Cy Quant assay to normalize the cell numbers (after seahorse experiment)

1) Immediately after the seahorse experiment, remove the media from the plates gently and freeze down the cells at -80. (you can keep the plate at -80 for a month).
2) Before the assay, perform multiple freeze-thawing (4 times).

3) Prepare the lysis buffer by diluting the lysis reagent by 20 times in ddH2O and dilute the Cy-Quant fluorescent reagent 400 times in the prepared lysis buffer.
4) Add 200 ul of this buffer on top of the thawed cells and pipette up and down.
5) Measure the fluorescence in 5 min at 485 nm ex and 530 nm emission.
    a. Select bottom read.
    b. Read all areas.

8.8 Mouse anti-viral kit Legend plex assay for serum samples from poly I:C and LPS peritoneal injection.

Reagents and materials

i. Samples
ii. Assay buffer
iii. Standard vial (Resuspend in 250 ul assay buffer)
iv. Matrix A (Resuspend in 5 ml assay buffer)
v. Pre-mixed beads (Ready to use)
vi. Detection antibodies (Ready to use)
vii. SA-PE (Ready to use)
viii. Wash buffer (20X)

Procedure

1. Sample dilution: 10X diluted in Assay buffer.
2. Bring all reagents to room temperature (20-25°C) before use.
3. Always keep the plate protected from light.
4. Standard for serum samples must be made in assay.
5. Load the standards in the first two columns of the 96 well plates. (see kit manual PLATE MAP, page 33)

a. For standard: 25 ul of Standard + 25 ul of Matrix

6. Load the samples to 96 well plates based on the replicates you planned. (see kit manual PLATE MAP, page 33)
    a. 25 ul of sample (5X) + 25 ul of assay buffer
7. Vortex the pre-mixed beads for one-minute before use.
8. Add 25 ul of pre-mixed beads to the diluted standards and samples and mix gently 3-5 times with pipette.
9. Seal the plate carefully and wrap it with foil to protect from light and shake at 250 rpm on the cell culture shaker for 2 hours at room temperature.
10. Centrifuge the plate for 1000 rpm for 5 minutes and flip the plate to remove the supernatant. Blot the plate very gently on paper towel to remove excess supernatant. (Watch for the pellet- don't tap hard if the pellet is dissociating from the bottom of the plate)
11. Wash the pellet with 200 ul of washing buffer. Here, just add the washing buffer on top of the pellet and incubate at RT for 1 min and centrifuge at 1000 rpm for 5 minutes. (Do not mix the pellet using pipette)
12. Add 25 ul of Detection Antibodies to all wells and mix the beads pellet with pipette gently 3 to 5 times.
13. Seal and shake the plate as before for 1 hr at RT at 250 rpm. (Protected from light)
14. After one hour, DO NOT CENTRIFUGE, add 25 ul of SA-PE directly to each well and shake similarly for 30 minutes.
15. Centrifuge the plate at 1000g for 5 minutes and resuspend the pellets in wash buffer with pipette and rune the assay in the pre-set template in the Attune Flow-cytometer.

16. After running the assay, export the FCS files and analyse in the legendplex™ cloud base analysis software (https://legendplex.qognit.com/user/login?next=home)

* Use clip tips for loading samples and premixed beads.

* refer to the following manual for reagent preparations and plate loading map, instrument set ups etc.):
https://www.biolegend.com/Files/Images/media_assets/pro_detail/datasheets/75354_Mu_Anti-Virus_Resp_FINAL_R2.pdf?v=20221013063042

8.9 Knockout mice generation

These mice were generated by Dr. Ali Abdul-Sater during his postdoc along with Dr. Christian W Schindler in 2012 at Columbia University. The generation entailed the genetic editing strategies Cre-LoxP and Frt/ Fleppe recombination system. In the generation, the respective IFN-I locus regions were flanked with LoxP, Frt or Lox5171 elements (Figs. 1). Neomycin and puromycin gazettes were also introduced into the locus. (Fig. 1) This was achieved by sequentially targeting embryonic stem cells (ES) with two targeting vectors TV1 and TV2. The element Lox5171is introduced for subsequent re-introduction of IFN-α subtypes to make knock-in mice. These ES cells were doubly targeted with TV1 and TV2 to introduce these elements and these targeted ES cells were used to generate heterozygous targeted mice. These ES cells were derived from 3.5 days age preimplantation embryo stage/ Blastocyst (Pluripotent) stage that can contribute to all embryonic tissues, including the germ cells in developing mice. The mouse from the embryo is chimeric, which was done in mouse 129 strain. These mice were then crossed with corresponding Cre/Fleppe mice to generate the above-explained genotypes. Then these mice were back crossed with C57BL/6 mice and intercrossed to yield the desired genotype in the C57BL/6 background.

They also confirmed the that these IFN-β^{only} mice are phenotypically not different from heterozygous littermates or WTs and breeding well.

9 APPENDIX C: ROLE OF INTENSITY ON EXERCISE-MEDIATED TRAINING OF THE IMMUNE RESPONSE.

Mayoorey M. Thasan, Amandeep Amandeep, and Ali A. Abdul-Sater*

9.1 Abstract

Inflammation is one of the earliest protective responses of the immune system. However, dysregulated inflammation is associated with serious human diseases, including cancer, cardiovascular diseases, diabetes and autoimmune disorders. Exercise is one of the external factors that can modulate inflammation. However, effects of exercise intensity on the exercise induced persistent effects on immune cells are not well studied. In this study, we employed an exhaustive exercise and a low-intensity moderate exercise protocol along with the previous high-intensity moderate exercise training. We hypothesised that the exhaustive exercise would increase the inflammation in BMDMs and high-intensity moderate exercise will dampen it while the low-moderate intensity will not have any effects. To study this, C57/Bl6 mice were exercised on a treadmill. Bone marrow-derived macrophages (BMDMs) from the exercised and sedentary mice were stimulated in-vitro with Lipopolysaccharide (LPS). Immunoblotting, Real-time PCR, Seahorse, Flow-Cytometric and ATAC-Seq (genome-wide chromatin accessibility) analyses were performed to evaluate persistent inflammatory, metabolic, and epigenetic changes in macrophages. We observed increased inflammation in BMDMs of mice undergone exhaustive exercise, but reduced inflammation in high-intensity moderate exercise. To study the mitochondrial adaptations, we measured mitochondrial oxidative stress and mitochondrial content. Interestingly, unlike we hypothesised both moderate intensity exercise protocols reduced the mitochondrial oxidative stress

and improved mitochondrial function and low-moderate exercise showed highly significant mitochondrial adaptations. We did not observe any significant changes in mitochondrial content. Additionally, ATAC-seq analysis showed differential chromosome accessibility in immune and metabolism associated regions (*ATACseq data- complex analysis is progress yet complete- data not shown*). Mechanistically, these observations suggest that our moderate exercise protocols lead to persistent adaptations in macrophages towards anti-inflammatory phenotype while exhaustive exercise might train them pro-inflammatory. Thus, our data suggest examining these adaptations in depth, will create more avenues in designing therapies or exercise mimicking pharmaceutical targeting to treat inflammatory diseases.

9.2 Introduction

Regular exercise is known to have beneficial effects on immune function. However, outcomes depend on a complex set of adaptations that happens in various tissues. Especially moderate-intensity exercise has been shown to positively impact infection risk and inflammatory diseases such as cardiovascular diseases, cancer, autoimmune diseases, etc.[77–80,81,82] On the other hand, High-intensity exercise is shown to course adverse effects.[66–68] Furthermore, there is a belief that high-intensity exercise impairs the immune response, leading to an “open window” for the risk of infection. The increased inflammatory response and impaired immune function observed following high-intensity exercise could be due to the body's inability for adaptation to heavy exertion. This may occur through acute functional impairment of various parts of the immune system. If the body has not adapted to the exertion, it will still be vulnerable to immune responses following high-intensity training. Indeed a study on very elite intense trainers reported that the illness rate in this population was less, supporting that the adaptation to the demand of intense training mitigates the negative outcomes.[93] However, there are inconsistencies in the study designs

regarding exercise models, intensity, and duration, and there is still a gap for reliable evidence to support this dogma. [95–97]

In 1914 Neiman et al. proposed a J-shaped model for infection risk vs. exercise intensity by summarizing studies that reported on Upper Respiratory Infection Risk (UTRI) in athletes involved in heavy training, ultramarathon events and moderate exercise training.[86,89] Although many studies with elite trainers challenged this model subsequently, numerous studies have supported that the health benefits are responsive to exercise dose .

Furthermore, prescribing generalized exercise therapies for patients and making them meet the exercise requirements are not always feasible. Especially patients with chronic diseases may have different physiological and immunological status. Therefore, designing precise exercise therapies or creating exercise mimic pharmaceuticals to treat or prevent various chronic diseases must be performed with caution. Thus, understating how the exercise mode and intensity impact these molecular adaptations will reveal valuable insights for designing therapies to treat a wide range of inflammatory diseases and chronic infections.

In this study, we employed different intensity treadmill exercise trainings on C57BL/6 mice to examine the effect of exercise intensity on exercise induced adaptations on bone marrow-derived macrophages. Mainly we focused on persistent inflammatory, immunometabolic and epigenetic adaptations. We employed RT-PCR to measure inflammatory gene expressions, flow cytometry to measure mitochondrial oxidative stress and membrane potential, seahorse analysis to measure cellular respiration and genome-wide chromatin accessibility assay (ATACseq) to evaluate adaptations in bone marrow derived macrophages upon multiple intensity exercises.

9.3 Hypothesis and Objectives

This project aims to study how different exercise programs of various intensities affect the long-lasting metabolic and epigenetic changes in BMDMs and how these adaptations affect the inflammatory response to future stimuli.

Objectives

1. How do various exercise intensities affect the persistent inflammatory, immunometabolic and epigenetic adaptations in BMDMs?

9.4 Methods

9.4.1 High-intensity moderate exercise training protocol (HME)

Female C57BL/6J (WT) mice aged six weeks were exercised. Sedentary group was rested in the cage and exposed to all environments as exercise mice. Both the exercised and the sedentary groups were first acclimatized to the five-lane treadmill for 5 days (Schneider-Electric) at 10m/min for the first 5 min and 20 m/min for the next 5 min (total 10 min) at 0° inclination. Exercise protocol was started following week with speed 20 m/min (0° inclination) 1 hr every day (5 days per week) and gradually increased (10% every week) up to 30 m/min and continued for 8 weeks. Mice were rested every weekend throughout exercise protocol.

9.4.2 Low-intensity moderate Exercise training protocol (LME)

Female C57BL/6J (WT) mice aged six weeks were exercised. Sedentary group was rested in the cage and exposed to all environments as exercise mice. Both the exercised and the sedentary groups were first acclimatized to the five-lane treadmill for 5 days (Schneider-Electric) at 10m/min for the first 5 min and 20 m/min for the next 5 min (total 10 min) at 0° inclination. Exercise protocol was started following week with speed 12 m/min (0° inclination) 45 min every day (5 days per week) and gradually increased (10% every week) up to 20 m/min and continued for 8 weeks. Mice were rested every weekend throughout exercise protocol. (Fig.1)

9.4.3 Cell culture and reagents.

Bone marrow derived macrophages (BMDMs) were prepared from the femur and tibia of mice and cultured in S+ media (RPMI 1640- Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Wisent), 2-Mercapthoethanol (Gibco), 1% L-Glutamine-Pyruvate-Penicillin-Streptomycin (Sigma), non-essential amino acids (Gibco) and 25% L929-conditioned media), as previously reported.[330] Lipopolysaccharide (LPS; Escherichia coli; serotype O26:B6) was obtained from Sigma. Red blood cell lysis buffer (Sigma), DPBS and PBS (Sigma)

9.4.4 Flow cytometry

For mitochondrial quality assays, BMDMs were induced with LPS (1 μg/ml and 5 μg/ml) for 6 hrs then stained for 10 min at 37°C either with 2.5 μM MitoSox (Thermo Fisher) and 40 μM mBBr (Thermo Fisher) made in FACS buffer to assess oxidative stress or 5 μM JC-1 made in FACS buffer (Thermo Fisher) to assess mitochondrial membrane potential. Similarly, mitochondrial mass measurement, treated BMDMs were stained with 50 nM Mitotracker™ Green in FACS buffer.[330] Cells were analyzed on an Attune NxT flow-cytometer (Thermo Fisher).

9.4.5 Bioenergetic analyses

Cellular oxygen consumption rate (OCR) and proton efflux rate (PER) were measured using the Seahorse XFe24 Analyzer (Agilent Technologies). 50,000 BMDMs were plated on Seahorse XF plates (Agilent) in S+ media and allowed to adhere overnight. Media was replaced with XF DMEM media pH 7.4 (Agilent) supplemented with glucose, glutamate and pyruvate, and incubated in CO_2 free incubator for at least 1 h prior to loading into the instrument. XFe24 sensor cartridges were hydrated overnight with XF Calibrant at 37°C. Various inhibitors/inducers were loaded to the ports depending on the assay.

For Mitochondrial stress test, Oligomycin (sigma), Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP; sigma), Rotenone (sigma), and Antimycin (sigma) were added

sequentially, as per manufacturer's instructions. For bioenergetics parameter calculations, all measurements were normalized as a percentage of the basal OCR. [331–333] Normalized the cell numbers using the Cy-Quant method.

For real time macrophage polarization, inducers such as LPS (100 ng/ml), LPS (100 ng/ml) + IFNγ (20 ng/ml) and IL4 (20 ng/ml) were injected through port A. PER values were measured for 6 hrs every five minutes and normalized to basal values prior stimulations.

9.4.6 Statistics

The results are presented as means with standard error of the mean (SEM). Significance is determined by a p-value of less than 0.05. Each experiment was conducted multiple times. N values indicated in the figure captions are biological replicates. When comparing two groups, an unpaired t-test is used. When comparing more than two groups, ANOVAs are conducted and followed by a post-hoc analysis, terky's, depending on the type of ANOVA. GraphPad Prism 9.4.1" (San Diego, CA, USA) was used to perform the statistical analysis for all the experiments.

9.5 Results

It is well known that moderate exercise can lower inflammation while reducing the risk for viral infections, particularly upper respiratory tract infections.[84,85,101,321,336–341] However, the mechanisms behind these effects are still under investigation. A growing body of literature is addressing this question. Our previous study showed a high-intensity moderate exercise training led to persistent changes that trained the BMDMs towards anti-inflammatory phenotype, which occurs via long-lasting metabolic and epigenetic adaptations. (**Chapter 4**) To examine the effect of exercise intensities on those persistent adaptations in BMDMs, we employed an exhaustive exercise (*data not shown-performed by a previous master's student*) and a low-intensity moderate exercise protocols along with the previous high-intensity moderate exercise training. We examined

the inflammatory, mitochondrial, metabolic, and epigenetic adaptations. We observed increased inflammation in BMDMs of mice undergone exhaustive exercise. This was assessed by measuring multiple inflammatory signalling pathway gene expression patterns using RT-PCR and immunoblotting. Epigenetic alterations were evident in both exercise intensities. (*ATACseq data- complex analysis is progress yet complete- data not shown*). To study the mitochondrial adaptations, we measured mitochondrial oxidative stress and mitochondrial content.

9.5.1 Mitochondrial oxidative stress is reduced in BMDMs following both high and low-intensity moderate exercise

Mitochondrial ROS plays important roles in inflammation and metabolic reprogramming in macrophage polarization.[363–365] We have shown in the previous study that long-term moderate exercise improves mitochondrial oxidative stress upon LPS-induced inflammation in BMDMs. Here we assessed the two different exercise intensities on mitochondrial ROS generation and the cellular reduced glutathione level and compared to sedentary mice. There was a significant increase in reduced glutathione levels and a decrease in mitochondrial ROS generation in exercised BMDMs. Interestingly, higher significance was observed for low-intensity moderate exercise protocol. This data suggests that even the lower-intensity moderate exercise program mediates mitochondrial adaptations in BMDMs, as we observed in the previous study.

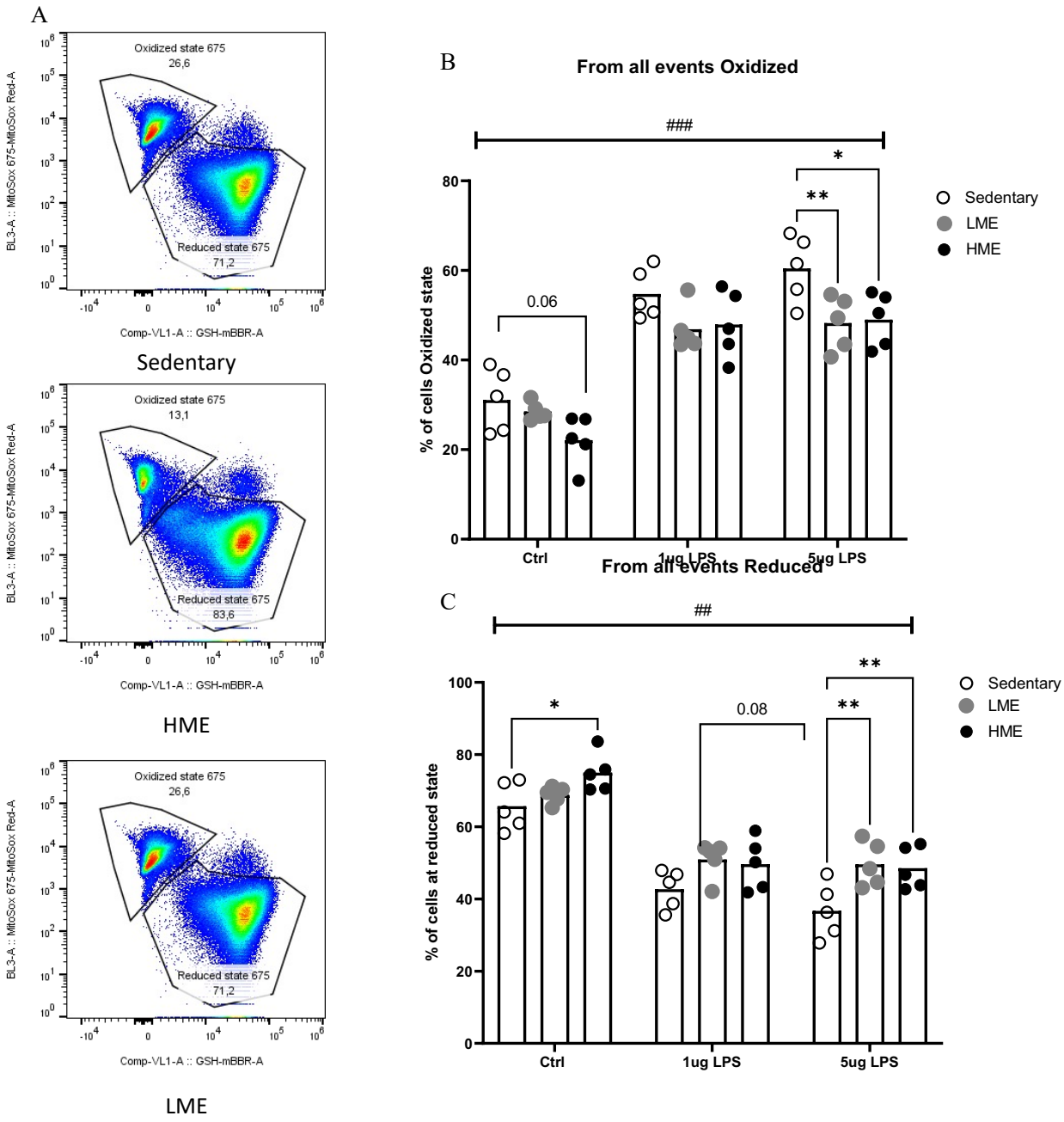


Fig. 1: **Long term Moderate-High intensity exercise improved mitochondrial (mt) health and content in bone marrow-derived macrophages**. BMDMs from both exercised and sedentary controls (C57 BL/6 (WT)) were stimulated with 1µg/ml and 5µg/ml LPS for 6 hours and mtROS, intracellular GSH levels and mt membrane potential were measured. A) representative graphs showing percentages of cell populations in oxidized and reduced states based on the mtROS levels and intracellular levels of GSH. B) Average percentages of cell populations in oxidized and C)

reduced state. D) representative graphs showing percentages of cell populations with depolarized and healthy mt membrane as measured by JC-1 dye. JC-1 aggregates indicate intact mt membrane potential, while JC-1 monomers indicate depolarized mt membrane potential. E) Average percentage of cells with healthy and F) depolarized mitochondria for different treatments as indicated. Stats: 2-way Anova, alpha: 0.05. (n=5), * Denotes the post-hoc analyses indicating a significant difference from multiple comparison. # denotes the overall statistical difference for intensity factor (P value) alpha: 0.05 $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.Each symbol represents an individual mouse. Statistical analysis was performed using 2 Way ANOVA with alpha: 0.05.

9.5.2 Mitochondrial metabolism is altered in BMDMs following both high and low-intensity moderate exercise

The metabolic switch between OHPHOS and glycolysis is important in macrophage phenotype conversion.[366–369] Here, we measured the glycolytic metabolism of naive, M1 and M2 polarized BMDMs to examine whether the mitochondrial adaptations following exercise are associated with metabolic reprogramming in the cells. Proton efflux rate (PER) was analyzed to assess glycolysis. BMDMs from both exercise groups showed a significantly larger shift in PER upon real-time M1 polarization than the sedentary group. The shift is more significant in low-intensity moderate exercise than in high-intensity moderate exercise. This may indicate that our high intensity moderate exercise leaning toward the line between high vs moderate exercise.

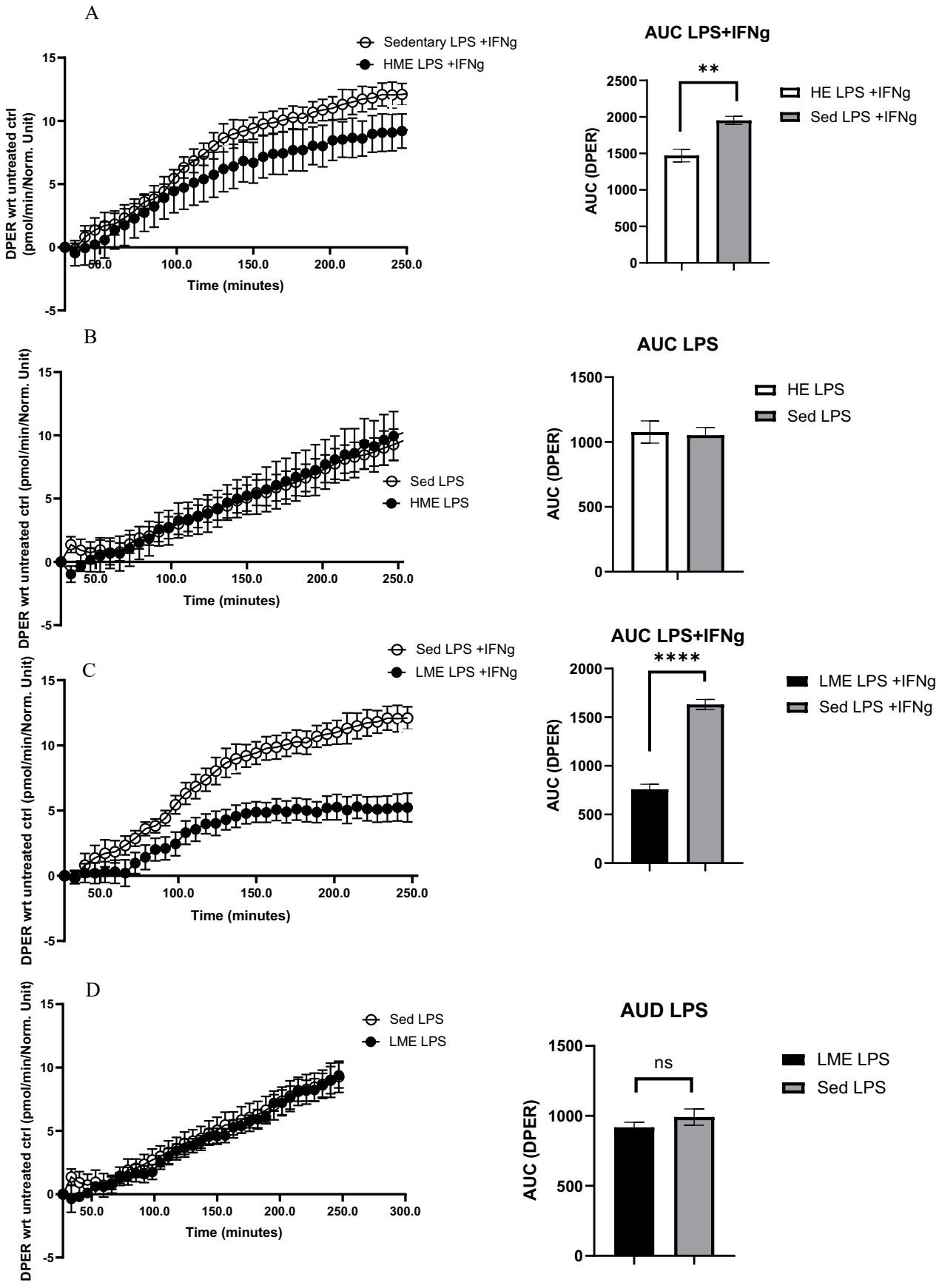

A
Sedentary LPS +IFNg
HME LPS +IFNg
DPER wrt untreated ctrl (pmol/min/Norm. Unit)
Time (minutes)
AUC LPS+IFNg
**
HE LPS +IFNg
Sed LPS +IFNg
AUC (DPER)
B
Sed LPS
HME LPS
DPER wrt untreated ctrl (pmol/min/Norm. Unit)
Time (minutes)
AUC LPS
HE LPS
Sed LPS
AUC (DPER)
C
Sed LPS +IFNg
LME LPS +IFNg
DPER wrt untreated ctrl (pmol/min/Norm. Unit)
Time (minutes)
AUC LPS+IFNg
****
LME LPS +IFNg
Sed LPS +IFNg
AUC (DPER)
D
Sed LPS
LME LPS
DPER wrt untreated ctrl (pmol/min/Norm. Unit)
Time (minutes)
AUD LPS
ns
LME LPS
Sed LPS
AUC (DPER)

Fig. 2: **Long term Moderate-High intensity exercise improved oxidative phosphorylation upon M2 polarization and reduced glycolysis switch in bone marrow-derived macrophages.** BMDMs from exercised and sedentary groups (C57 BL/6 (WT)) were stimulated with M1 and M2 stimulation media (M1:100 ng/ml LPS, 20 ng/ml IFN-γ and M2: IL4 20 ng/ml). Cells were stimulated for 24hrs for the OCR measurement and PER was measured real-time for 6 hrs while the macrophages are polarizing. OCR was assessed using mitostress assay G) Changes in PER values upon stimulation that represents the metabolic shift (DPER). Data was normalized to baseline (left panel) Area under the curve for the PER curves (right panel). Stats: t-test, nonparametric with alpha: 0.05 $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

9.6 Discussion

It is well known that moderate regular exercise can promote systemic and tissue-level anti-inflammatory effects, providing health benefits in various chronic diseases and infections. [17,107,314,315,317,320–322,325,372–376]. On the other hand, high-intensity exercise is shown to cause adverse effects.[83–85] The primary purpose of this study is to examine different exercise intensities.

Exercise causes systemic changes in exerkines, including metabolites, proteins, lipids, and inflammatory mediators, which circulate to reach lymphoid tissues, such as the bone marrow.[9] These biomolecules together with energy demand and stress signals can contribute to metabolic and epigenetic reprogramming and alter mitochondrial quality and function in resident and bone marrow-derived immune cells. This study demonstrates that moderate-intensity long-term exercise programs promote mitochondrial health and function.

Indeed, we observed reduced oxidative stress and improved OXPHOS in exercised BMDMs. Mitochondria, now known as an immune organelle, is a key regulator of immune signalling.[383–387] Thus, persistent mitochondrial adaptations could also play roles in the immunometabolic reprogramming and phenotype conversion of BMDMs following exercise.

In addition, it is interesting that lower-intensity moderate exercise showed higher significance for both oxidative stress and mitochondrial function-associated adaptations. Furthermore, reduction

in glycolysis switch upon M1 polarization in BMDMs implies a metabolic adaptation in BMDMs following exercise. This metabolic switch was larger in lower intensity moderate exercise. This could indicate that our HME protocol was close to the high intensity margin.

Combined with increased proinflammatory markers in BMDMs of mice performed exhaustive excise and epigenetic alterations observed for both high and low-intensity moderate exercise, this study shows macrophage memory or trained effects vary depending on the exercise intensity. Exhaustive exercise could train the macrophages to be more proinflammatory, while long-term moderate exercise trains them towards anti-inflammatory state. However, further studies examining more exercise intensities and other mechanistic parameters are necessary to understand the mechanisms more precisely.